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Detection of WSSV in cultured shrimps, captured brooders, shrimp postlarvae and water samples in Bangladesh by PCR using different primers

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Abstract

Detection of whitespot syndrome virus (WSSV) in cultured shrimps (*Penaeus monodon*), *P. monodon* brooders, shrimp postlarvae and water samples in Bangladesh was carried out by Polymerase chain reaction (PCR) using five different pairs of primers yielding amplicons ranging from 310 to 2310 bp. The primer pairs used in this study are designated as KF 1–2 (2310 bp), Lo 1–2 (1447 bp), Lo 5–6 (775 bp), IK 1–2 (486 bp) and IK 3–4 (310 bp). Out of 42 cultured shrimp tested, 36 were positive for WSSV, with 18 by non-nested reaction and 18 by nested reaction. In non-nested reaction only one shrimp sample showed positive for WSSV with all the five primer pairs, four showed positive with primer pairs Lo 1–2, Lo 5–6, IK 1–2 and IK 3–4, two showed positive with primer pairs Lo 5–6, IK 1–2 and IK 3–4, 2 positive with primer pairs IK 1–2 and IK 3–4 and nine showed positive with primer pairs IK 3–4 only. In nested reaction all the 18 samples showed positive with primer pairs IK 1–2 and IK 3–4. All the five brooders were positive for WSSV by non-nested PCR with primers IK 3–4 and only one was positive by primers yielding higher fragments. Out of 18 post larvae tested, two were positive by nested reaction using IK 1–2 and IK 3–4. Among 12 water samples analysed, three showed positive in non-nested reaction with primers IK 3–4 only and five showed positive in nested reaction with primers IK 1–2 and IK 3–4. All the PCR products were further confirmed by dot blot hybridization using biotinylated probe.

Keywords: WSSV; PCR; Dot blot hybridization; Primers; Cultured shrimps; Postlarvae

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1. Introduction

White spot syndrome disease continues to be the most serious threat associated with penaeid shrimps in all the shrimp growing countries globally and the Asian countries in particular (Inouye et al., 1994, 1996; Nakano et al., 1994; Cai et al., 1995; Wongteerasupaya et al., 1995; Karunasagar et al., 1997; Park et al., 1998; Magbanua et al., 2000; Hossain et al., 2001a). WSSV has been reported to affect several commercially important species of penaeid shrimps (Takahashi et al., 1994; Chou et al., 1995; Flegel, 1997; Karunasagar et al., 1998; Wang et al., 1999). WSSV has been detected in a wide range of wild crustaceans including penaeid and non-penaeid shrimps, crabs, lobsters, etc. (Lo et al., 1996a,b; Peng et al., 1998; Otta et al., 1999; Hossain et al., 2001b). It has also been found in association with other shrimp viruses such as monodon baculovirus and hepatopancreatic parvovirus (Otta et al., 2003; Umesha et al., 2003). In Bangladesh, white spot syndrome was first recorded in Cox's Bazar in 1994 (Larkins, 1995) and since then the shrimp farming industry in Bangladesh has been seriously affected by WSSV (Paul, 1997) resulting in substantial loss. Various diagnostic procedures have been developed for detection of WSSV. These include histopathological techniques (Wongteerasupaya et al., 1995; Supamattaya and Boonyaratpalin, 1996) in situ hybridization using gene probes (Durand et al., 1996; Wongteerasupaya et al., 1996) immunological methods such as Nitrocellulose-enzyme immunoblot (Nadala and Loh, 2000) and western blot techniques (Nadala et al., 1997; Hameed et al., 1998) and more recently polymerase chain reaction based methods (Lo et al., 1996a; Kim et al., 1998).

The level of sensitivity with the use of different DNA based techniques seems to vary. This is observed in a recent study of identification of white spot syndrome virus latency-related genes by use of a microarray (Khadijah et al., 2003). However, from the practical point of view, among the various diagnostic techniques, PCR provides a high degree of sensitivity and specificity in detection of WSSV (Lightner, 1996). A number of primers have been designed for PCR detection of WSSV by various workers. Lo et al. (1996a) designed three sets of primers based on sequence of cloned WSSV Sal I 1461 bp DNA fragment. The primers 146F1/146R1 gives an amplicon of 1447 bp while primers $146F_2/146R_2$ and $146F_4/146R_3$ give amplicons of 941 and 775 bp, respectively. Another set of primers was designed by Wongteerasupaya et al. (1996) based on the sequence of a specific probe yielding a 294 base pair fragment specific for WSSV DNA. Takahashi et al. (1996) developed a primer pair which amplified a 643 bp fragment of RV-PJ of Japan and designated the primers as PJ1 and PJ2. Kim et al. (1998) designed two DNA oligonucleotide primers named IF and IR based on sequence data of WSSV DNA which gives an amplicon of 365 bp. Nunan et al. (1998) designed two sets of primers based on sequence of WSSV genome. The first set (N/L primers) designated as 6581 and 7632 amplifies a 750 bp sequence of WSSV DNA while the second set of primers designated as 9987 and 9988 amplifies a 500 bp internal sequence to the first set of primers. Kasornchandra et al. (1998) used two oligonucleotide primers designated 102 F1 and 102 R1 which amplifies a 520 bp fragment of white spot virus genomic DNA. Magbanua et al. (2000) used a set of DNA oligonucleotide primers originally designed by Tapay et al. (1999) which gives an amplicon of 217 bp. Hossain et al. (2001b) used two sets of primers, designated IK 1–2 and IK 3–4 that were based on sequence from Gene Bank accession no. U50923 and giving an amplicon of 486 and 310 bp,

respectively. The specificity and sensitivity of a PCR reaction depends on the type of primers used. The sensitivity of PCR increases with decrease in size of amplicon yielded by a primer pair (Lo et al., 1996a; Hossain et al., 2001b). Against this background, our study was undertaken to evaluate the sensitivity of different primer pairs in detection of WSSV in shrimps, postlarvae and water from various ponds and ghers in Bangladesh.

2. Materials and methods

2.1. Sample collection

Shrimp samples were collected from 15 culture ponds and 27 culture ghers (enclosures of low lying land by dikes) in Cox's Bazar (Southeast Bangladesh) and the Khulna area. Gills were aseptically removed from the shrimps and were preserved in tubes containing 70% alcohol. Pleopods were collected from *Penaeus monodon* brooders (80–120 g) from various hatcheries in the Cox's Bazar region and were placed in 70% alcohol. Postlarval samples were collected from hatcheries at Cox's Bazar, Technaf (SE Bangladesh) and Bagerhat (W Bangladesh) region and put into 70% alcohol. Water samples were collected from various shrimp ponds, ghers, rivers, canals and sea at Cox's Bazar and Khulna. All the samples were brought to Mangalore, India for further analysis. The period of sampling was from May 2000 to June 2000.

2.2. WSSV DNA extraction

WSSV DNA extraction was carried out as described by Hossain et al. (2001b). Approximately 30–50 mg of gill/pleopod tissue of *P. monodon* and 20–30 postlarvae of *P. monodon* were homogenized individually with 1.5 ml TESP buffer (50 mM Tris–HCl, pH 8.5, 10 mM EDTA, 100 mM NaCl, 1 mM Phenyl Methyl Sulfonyl Fluoride) in a disposable UV sterilized polythene sachet and then transferred to a microfuge tube. Four μl Triton X-100 was added to each tube and incubated for 30 min at 45 °C. The sample was then centrifuged at 1500 × *g* for 10 min at 4 °C in a refrigerated centrifuge (Remi C-24, Remi Instruments, India) and the supernatant fluid was transferred to another microfuge tube and recentrifuged at 16,300 × *g* for 30 min at 4 °C. The pellet was then suspended in 400 μl TESP buffer and centrifuged at 16,300 × *g* for 30 min at 4 °C. The supernatant fluid was discarded and the pellet was finally dissolved in 50 μl of TESP. In case of water samples, 9 ml was taken in six microfuge tubes each containing 1.5 ml and centrifuged at 16,300 × *g* for 30 min at 4 °C. The pellets were pooled in one tube and then 4 μl of Triton X-100 was added and incubated at 45 °C for 30 min. This was then centrifuged at 16,300 × *g* for 30 min, and the pellet was suspended in 400 μl TESP and centrifuged at 16,300 × *g* for 30 min at 4 °C. Finally the pellet was resuspended in 50 μl TESP.

2.3. PCR primers

Five sets of primer pairs namely Lo 1–2, Lo 5–6, IK 1–2, IK 3–4 and IK 5–6 and KF 1–2 were used in the current study. The primers Lo 1–2 corresponded to primers 146F1

Table 1
Detection of WSSV in *P. monodon* from various ponds and ghers of Bangladesh by PCR

Sampling area Pond (P)/Gher (G)	Stocking density (per m^2)	Days of culture (DOC)	Weight (g)	Gross sign	Mortality	Reaction in non-nested PCR				Reaction in nested PCR IK1–2 → IK3–4
						Lo 1–2	Lo 5–6	IK 1–2	IK 3–4	
Cox's Bazar										
P1	4–5	50	12	H, MS	–	–	–	–	+	NA
P2	4–5	42	10	WS	+	–	+	+	+	NA
P3	7–8	62	15	WS, RB	+	+	+	+	+	NA
P4	7–8	60	14	WS	+	+	+	+	+	NA
P5	4–5	55	14	H	–	–	–	–	+	NA
P6	7–8	65	15	WS	+	+k	+	+	+	NA
P7	3–4	52	14	H	–	–	–	–	–	+
Khulna										
P8	2–3	38	8	H	–	–	–	–	–	+
P9	2–3	60	11	H	–	–	–	–	–	+
P10	2–3	60	15	MS	–	–	–	–	+	NA
P11	1–2	62	19	H	–	–	–	–	–	+
P12	2–3	58	14	MS	–	–	–	–	+	NA
P13	1–2	55	9	H	–	–	–	–	–	+
P14	1–2	58	15	H	–	–	–	–	–	–
P15	3–4	60	12	WS, RB	+	+	+	+	+	NA
Cox's Bazar										
G1	1–2	60	18	H	–	–	–	–	+	NA
G2	1–2	55	17	H	–	–	–	–	–	–
G3	2–3	67	18	H, MS	–	–	–	–	+	NA

G4	2–3	48	14	H	–	–	–	–	+	NA
G5	1–2	43	12	H	–	–	–	–	–	+
Khulna										
G6	1–2	87	26	H	–	–	–	–	–	–
G7	1–2	60	12	MS, TR	–	–	–	+	+	NA
G8	1–2	82	25	H	–	–	–	–	–	+
G9	1–2	79	24	H	–	–	–	–	–	+
G10	1–2	83	20	H	–	–	–	–	–	+
G11	1–2	68	15	MS, PG	–	–	–	–	+	NA
G12	1–2	72	17	H	–	–	–	–	–	–
G13	2–3	55	8	MS, BG	+	–	–	+	+	NA
G14	1–2	79	30	H	–	–	–	–	–	+
G15	1–2	66	18	H	–	–	–	–	–	–
G16	2–3	74	16	WS	+	–	+	+	+	NA
G17	1–2	90	18	H	–	–	–	–	–	+
G18	2–3	85	13	H	–	–	–	–	–	+
G19	2–3	92	21	H	–	–	–	–	–	+
G20	1–2	70	18	H	–	–	–	–	–	+
G21	1–2	55	11	H	–	–	–	–	–	+
G22	1–2	95	30	H	–	–	–	–	–	+
G23	1–2	92	28	H	–	–	–	–	–	–
G24	1–2	76	25	H	–	–	–	–	+	NA
G25	1–2	96	28	H	–	–	–	–	–	+
G26	2–3	79	20	H	–	–	–	–	–	+
G27	2–3	82	18	WS	+	+	+	+	+	NA

H—healthy; MS—minor white spot; WS—white spot; RB—reddening body; TR—tail rot; PG—pinkish gill; BG—black gill; NA—not applicable; k—also positive by Korean fragment; k—positive by primer KF 1–2.

and 146R1 and Lo 5–6 corresponded to 146F4 and 146 R3 as described by Lo et al. (1996a). The primers named IK 1–2 (486 bp), IK 3–4 (310 bp) and IK 5–6 (210 bp) were based on sequence from GenBank Accession No. U 50923 as described by Hossain et al. (2001b). The primers KF 1 and KF2 were designed based on the sequence from GenBank Accession No. U92007 and bind to position 49–61 and 2338–2358 of this sequence to amplify fragment of 2310 bp.

2.4. PCR protocol

PCR reactions were carried out in 50 μl of reaction mixture that consisted of 5.0 μl of Taq Polymerase Assay Buffer (20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 50 mg ml^{-1} nuclease free Bovine Serum Albumin) 1.0 μl (0.5 μg) of each primer, 1.0 μl (200 μM) dNTP mix, 3.0 μl of template DNA and 0.60 μl (2.25 units) Taq DNA polymerase and made up to volume using PCR grade distilled water.

For nested (2 step) PCR, 5.0 μl of the first step reaction mixture was added to the PCR cocktail. The DNA extracted from WSSV infected *P. monodon* was used as the positive control. DNA from a WSSV negative postlarvae and sterile distilled water was used as the negative control. Both positive and negative controls were added at 1 μl concentrations to the PCR cocktail. The PCR was performed in a thermocycler (MJ Research, USA) for 30 cycles each cycle consisting of three steps: denaturation of target DNA at 94 °C for 1 min, annealing of primers at 55 °C for 1 min and extension of primers at 72 °C for 2 min. The programme included an initial delay of 5 min at 94 °C and a final delay of 5 min at 72 °C. 20 μl of PCR product was mixed with 5.0 μl of loading buffer and subjected to electrophoresis in 0.8% agarose gels for products amplified by primers Lo 1–2, Lo 5–6 and KF 1–2 and 1.5% agarose gels for products amplified by primer IK 1–2 and IK 3–4. Ethidium bromide was added at a concentration of 0.5 μg/ml. The gels were observed and photographed using gel documentation system (Herolab, Germany).

2.5. Dot blot hybridization

DNA extracted from gills of *P. monodon* naturally infected with WSSV was amplified using primer pair IK 1–2. Using the product of this reaction, nested PCR was performed using primer pair IK 5–6. This product was purified using Concert PCR purification kit (Gibco, USA) and labeled with biotin 14-dATP using Nick Translation kit (Gibco) as per manufacturers instruction. The labeled product was purified again using Concert PCR purification kit and used as a probe in dot blot hybridization assays. The PCR products obtained from cultured shrimp, brooders, postlarvae and water samples were boiled for 10 min in a water bath and snap cooled on ice. Two microliters of each of the samples was spotted on to a nylon membrane (Nytran, NY 12 N, Schleicher and Schuell, Germany) and blotting was done as described by Dyson (1994). The DNA on the membrane was immobilized by UV crosslinker (UVC 500, Hoefer, USA) and the hybridization assay was performed as described by Rashtchian and Mackey (1992). The hybridized probe was detected using streptavidin-alkaline phosphatase conjugate (Bangalore, Genei, Bangalore) and chromogenic substrate following the manufacturer's protocol.

3. Results

From Table 1, it can be seen that the presence of WSSV in cultured shrimps is quite high with 36 of 42 samples analysed showing positive either in non-nested or nested PCR reaction. A total of seven ponds and five ghers were sampled in the Cox's Bazar region whereas eight ponds and 22 ghers were sampled in Khulna region. All the pond samples from the Cox's Bazar region showed positive for WSSV with 6 (P1 to P6) being positive in non-nested PCR and 1 (P7) by nested PCR. In case of gher samples, three (G1, G3, G4) were positive by non-nested PCR and one (G5) was positive by nested PCR. In the Khulna region, out of eight ponds sampled, shrimps from seven ponds (P8–P14) were apparently healthy and only one pond (P15) had gross signs of white spot. The PCR results indicate that four ponds (P8, P9, P11 and P13) were positive only by nested PCR whereas three were positive by non-nested PCR. Ponds P10 and P12 showed positive reaction only with primers IK 3–4 while P15 showed positive with primers Lo 1–2, Lo 5–6, IK 1–2 and IK 3–4. Out of 22 ghers sampled in the Khulna region, shrimps from 20 ghers were either healthy or showing minor spots and other clinical signs like black gill, tailrot, etc. Only two ghers G16 and G27 had shrimps showing gross clinical signs of white spot. PCR results show that samples from G16 and G27 were positive for WSSV with all the primers except KF 1–2 whereas samples from G7, G11, G13 and G24 were positive only with primer IK 1–2 and IK 3–4. Samples from G8 to G10, G14, G17 to G22, G25, G26 were positive only by nested PCR using IK1–2 and IK 3–4 and samples from G6, G12, G15, G23 were negative for WSSV with all the primers.

It is also seen from Table 1 that animals which had conspicuous white spots or those from ponds showing mortality showed PCR positivity with primers yielding higher amplicons, i.e. Lo 1–2 and Lo 5–6. In apparently healthy animals or in animals with minor white spots, PCR was positive only with primers yielding amplicons of small sizes, i.e. IK 1–2 and IK 3–4. One gher (G13) from the Khulna region did have mortalities and samples showed minor spots also. However, the animals showed black gills suggesting that the cause of the mortalities may not be due to white spot disease. The results from Table 2 show that all the five brooders were positive for WSSV in non-nested reactions

Table 2
Detection of WSSV in *P. monodon* brooders and postlarvae from Bangladesh by PCR

Sampling area/hatcheries	Number examined	No. of positive by non-nested PCR				No. of positive by nested PCR
		Lo 1–2	Lo 5–6	IK 1–2	IK 3–4	IK 1–2 to IK 3–4
Brooders						
Cox's Bazar	5	1k	1	1	5	NA
Postlarvae						
Cox's Bazar	9	0	0	0	0	2
Technaf	7	0	0	0	0	1
Bagerhat	2	0	0	0	0	0
Postlarvae total	18	0	0	0	0	3

k—positive by primer KF 1–2; NA—not applicable.

Table 3
Detection of WSSV in water samples from Bangladesh by PCR

Sampling area/type	Number examined	No. of positive by non-nested PCR				No. of positive by nested PCR
		Lo 1–2	Lo 5–6	IK 1–2	IK 3–4	IK 1–2 → IK 3–4
Khulna						
Shrimp pond	2	0	0	0	1	1
Shrimp gher	2	0	0	0	0	1
River	1	0	0	0	0	0
Canal	1	0	0	0	0	1
Cox's Bazar						
Shrimp pond	2	0	0	0	2	NA
Shrimp gher	1	0	0	0	0	1
River	1	0	0	0	0	0
Hatchery	1	0	0	0	0	1
Sea	1	0	0	0	0	0
Total	12	0	0	0	3	5

k—positive by primer KF 1–2; NA—not applicable.

with all five showing positive with primer IK 3–4 and only one showing positive with all the other primer pairs.

The PCR results for postlarvae (Table 2) indicate that the larvae did not have a high load of WSSV; none of them were positive for WSSV by non-nested PCR out of 18 samples tried. Only three were positive by nested PCR (IK 1–2 and IK 3–4).

As shown in Table 3, out of 12 water samples collected from various culture ponds, ghers, hatchery, rivers and sea, only three pond samples (one from Khulna and two from

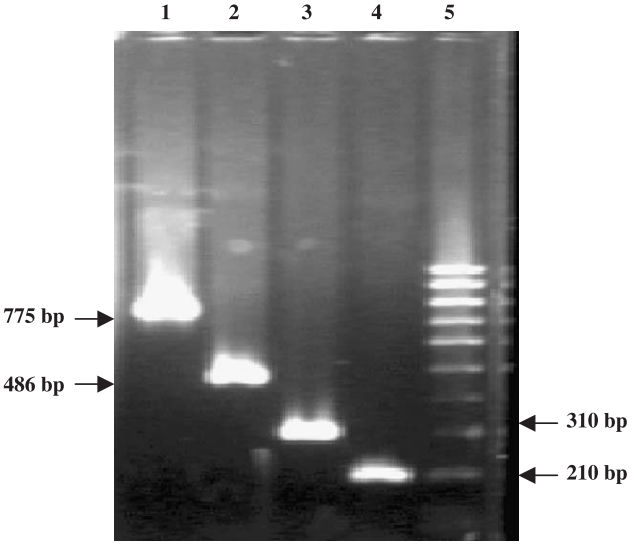

Fig. 1. PCR detection of WSSV in *P. monodon* using different primer pairs. Lanes: (1) Primers Lo 5–6 (775 bp), (2) IK 1–2 (486 bp), (3) IK 3–4 (310 bp), (4) IK 5–6 (210 bp), (5) 100 bp molecular weight marker.

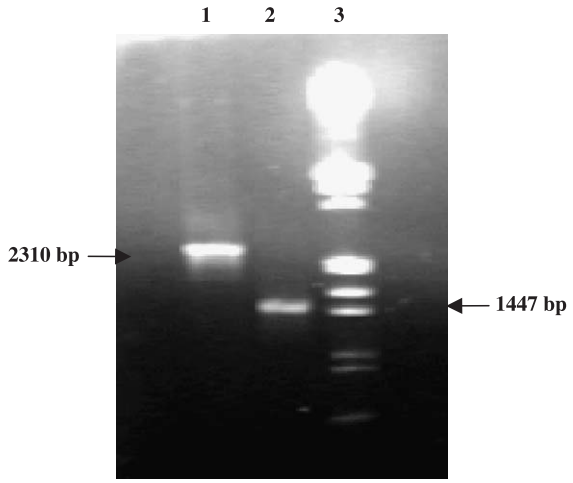

Fig. 2. PCR detection of WSSV in *P. monodon* using primer with bigger amplicons. Lanes: (1) Primers KF 1–2 (2310 bp), (2) Lo 1–2 (1447 bp), (3) molecular weight marker (λDNA/*Eco* RI *Hind* III double digest).

Cox's Bazar) were positive for WSSV by non-nested PCR with IK 3–4 whereas two gher samples (one each from Khulna and Cox's Bazar), one canal sample from Khulna and one hatchery sample from Cox's Bazar were positive for WSSV by nested PCR (IK 1–2 and IK 3–4). However, the sea water samples and river water samples from Khulna and Cox's Bazar were negative both by nested and non-nested PCR. Figs. 1 and 2 shows the PCR results of various samples using different primers. All the PCR products were confirmed as originating from WSSV by hybridizing with 14-dATP biotin labeled probe and representative date is shown in Fig. 3.

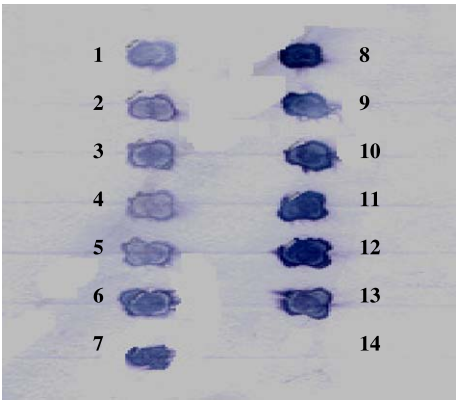

Fig. 3. Confirmation of WSSV PCR products of *P. monodon*, *P. monodon* brooder, *P. monodon* postlarvae and water by dot blot hybridization with 14-dATP biotinylated probe. Lanes: (1–5) *P. monodon* (non-nested IK 1–2 products), (6) *P. monodon* brooder (non-nested IK 1–2), (7) Positive control (non-nested Lo 1–2 product), (8–10) *P. monodon* larvae (nested IK 3–4 products), (11–13) water (nested IK 3–4 products), (14) negative control.

4. Discussion

The results of this study bring out the variability in the results of PCR analysis depending on the primers used. Different workers have reported use of different primers for detection of WSSV. The OIE Diagnostic Manual for Aquatic Animal Diseases (OIE, 2003) recommends the use of primers described by Lo et al. (1996a) yielding amplicon sizes of 1447 and 941 bp for diagnostic PCR for WSSV. The OIE Manual also recommends the use of PCR for both confirmatory diagnosis as well as for screening of infection status. The results of our study show that the primers described by Lo et al. (1996a) yielding amplicons of 1447 and 775 bp are good for confirmatory diagnosis of whitespot disease, but not for screening purposes. All animals with clear white spots with or without reddishness and mortality were positive with primers yielding higher amplicons, i.e. KF 1–2 (2310 bp), Lo 1–2 (1447 bp) and Lo 5–6 (775 bp). However, animals with minor spots or apparently healthy animals were positive by either non-nested PCR with primers yielding smaller amplicons i.e. IK 3–4 (310 bp) or by nested PCR (IK 1–2 and IK 3–4). Hence, it is clear that the sensitivity of detection and number of positivity increases as the amplicons size of the primers used decreases. Our earlier studies (Hossain et al., 2001b) clearly show that when the viral load is low, the chances of getting positive results are better with primers yielding smaller amplicons. Park et al. (1998) reported that PCR for WSSV with primers specific to RV-PJ (Takahashi et al., 1996) yielding an amplicon of 643 bp gave a positive reaction whereas PCR with primers specific to WSSV designed by Lo et al. (1996a) showed a negative reaction. In our study also it has been found that a number of samples from healthy animals from ponds as well as from ghers showed positive for WSSV with primers IK 3–4 but were negative with primers Lo 1–2, Lo 5–6 and IK 1–2.

The results of PCR analysis for broodstock and post larval samples also indicate the variability in positivity depending on the type of primers used. 100% positivity has been seen in broodstock samples when PCR using primers IK 3–4 was done whereas only 20% are positive when PCR using primers yielding bigger amplicons (KF 1–2/Lo 1–2/Lo 5–6/IK 1–2) was performed. Analysis of postlarval samples indicate that only 16.66% (3/18) are positive for WSSV by nested PCR reaction using primer IK 1–2 and IK 3–4. According to Hossain et al. (2001a), in the case of screening for carrier state infection as in brooders and postlarvae, there is a need to use primers yielding smaller amplicons. Chakraborty et al. (2002) have reported that out of 89 crustacean samples examined for presence of WSSV, none were positive with primer pairs Lo 1–2 whereas 43 were positive when primer pair IK 3–4 was used and one showed a positive reaction with primer pair IK 1–2 in non nested PCR. Hossain (2001) reported that 23.34% of postlarvae from various hatcheries along the west coast of India were positive for WSSV with primer pair IK 3–4 whereas only 14% were positive with primer pair IK 1–2.

From this study it can be clearly seen that sensitivity of PCR detection of WSSV depends on the size of amplicon generated which in turn depends on the type of primers used. The primers should be chosen taking into consideration the purpose of diagnosis. If PCR is used for confirmatory diagnosis of overt disease, primers yielding larger fragments can be used but if the purpose of the PCR is to screen for WSSV and in cases where a low

virus load is expected, use of primers yielding smaller amplicons would give more accurate results.

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