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Estimation of prevalence of white spot syndrome virus (WSSV) by polymerase chain reaction in *Penaeus monodon* postlarvae at time of stocking in shrimp farms of Karnataka, India: a population-based study

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ABSTRACT: White spot disease (WSD) is at present the most serious viral disease affecting cultivated shrimp species globally. The causative agent, white spot syndrome virus (WSSV), is extremely virulent, has a wide host range and can also be transmitted from broodstock to their offspring. The shrimp postlarvae (PL) act as asymptomatic, latent carriers of the virus, and stocking of WSSV-infected PL has been reported as a risk factor for WSD outbreaks in culture ponds. However, there is no population-based study on WSSV prevalence in PL of shrimp. The present manuscript documents the approaches and the results in the estimation of prevalence of WSSV in PL populations of *Penaeus monodon* at the time of stocking. A maximum of 300 PL from each of the 73 batches of PL stocked at various farms in the west coast of India during September 1999 to January 2000 were tested for the presence of WSSV by 2-step nested PCR. Thirty-six (49%) of the 73 batches tested positive for WSSV either by 1-step alone (3 batches) or after 2-step nested PCR (33 batches). Sub-samples of 5 PL each or 1 PL each tested to quantify the proportion of infected PL within batches showed that WSSV prevalence was very high in 1-step PCR-positive batches and low in 2-step PCR-positive batches. The study also showed that appropriate sampling and sample size were major factors in determining the prevalence of WSSV in PL populations, underlining the need for testing large samples of PL to reduce errors from falsely negative results.

KEY WORDS: WSSV · PCR · *Penaeus monodon* · Postlarvae · Prevalence · Aquatic epidemiology

INTRODUCTION

Ever since it was first reported following outbreaks in Japan and China (Nakano et al. 1994, Takahashi et al. 1994, Chou et al. 1995), white spot disease (WSD) has proved to be the most serious epidemic in cultured shrimp throughout Asia (Wang et al. 1995, Wongteerasupaya et al. 1995, Lo et al. 1996a, Flegel 1997, Park et al. 1998, Sudha et al. 1998, Magbanua et al. 2000) and, more recently, in the Americas (Calderon et al. 2000). The causative agent, white spot syndrome virus (WSSV) is a bacilliform, double-stranded DNA virus (Wang et al. 1995, Lightner 1996). The taxonomic position of the virus is yet to be approved by the Interna-

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tional Committee on Taxonomy of Viruses (ICTV). The entire genome sequence of the virus has recently been reported. It is a 292.976 kb long, circular, double-stranded DNA representing an entirely new virus family, and a new family name '*Nimaviridae*' has been proposed (van Hulten et al. 2001).

WSSV has a wide host range, affects almost all species of cultured shrimp (Lo et al. 1996b) and is extremely dangerous because of its ability to cause mass mortalities in culture ponds. Hence, it is regarded as a C-1 category pathogen (Lotz 1997). There is evidence of WSSV infection in the reproductive tissue of male and female *Penaeus monodon* broodstock (Lo et al. 1997, Mohan et al. 1997) and the postlarvae (PL) from infected *P. monodon* broodstock have been found to be infected with WSSV (Lo & Kou 1998). Hence, *P. monodon* PL are considered as a major entry route for WSSV into culture ponds (Limsuwan 1997, Flegel & AldaySanz 1998, Mushiake et al. 1999). Stocking of 1-step PCR-positive PL batches has also been associated with disease outbreaks and crop failure (Withyachumnarnkul 1999).

WSSV in India was first reported in 1994 along the east coast (Mohan et al. 1998) and quickly spread to the west coast and all the culture areas of the country (Shankar & Mohan 1998), causing severe economic losses. Since then, it has become endemic to all the intensive as well as artisanal culture areas, with a pattern of cyclic epidemics.

The rapid and extensive spread of WSSV has been attributed to the movement of infected PL and frozen shrimps (Momoyama et al. 1994, Nakano et al. 1994, Nunan et al. 1998). Due to the pandemic nature of the disease, much emphasis has been given to prevention and early diagnosis of WSSV. For all these reasons, in recent years, screening of PL or broodstock for WSSV by PCR has been proposed to the shrimp-farming industry as a major component of disease-management strategies (Limsuwan 1997, Lo & Kou 1998).

Several WSSV-diagnostic PCR methods have been developed for screening and early detection of the disease (Lightner 1996, Lo et al. 1996a, Takahashi et al. 1996, Kim et al. 1998, Maeda et al. 1998). A 2-step (nested) PCR (Lo et al. 1996b) has been developed into one of the most powerful tools for detecting the pathogen, and this has been used to screen carriers, shrimp larvae and spawners and to monitor WSSV in cultured shrimp (Lo et al. 1998).

The aim of the present investigation was to estimate the prevalence of WSSV in *Penaeus monodon* PL at the time of stocking on the west coast of India. The results presented in this paper are part of a larger epidemiological study on WSD. The present manuscript is the first document on WSSV prevalence in PL from a population-based study in which statistically based samples of PL batches were considered over a period of time. Besides its epidemiological relevance, the paper also identifies and discusses some practical issues regarding the methodology for effective screening of PL for WSSV by PCR.

MATERIALS AND METHODS

PL samples. *Penaeus monodon* PL samples were collected at the time of stocking from shrimp farms in Kundapur (13° 36′ to 13° 42′ N, 74° 40′ to 74° 44′ E), Karnataka, along the west coast of India. As part of the longitudinal observational study, a random sample of 100 shrimp ponds was selected, representing the shrimp farms around the Kundapur estuary. Of these, 70 ponds were enrolled in the investigation and data were collected on the PL batches stocked in each pond. A total of 73 stocking events took place in these ponds between September 1999 and January 2000. Farms were visited at each stocking event and a sample of 500 PL was collected by pooling scoop samples from all the delivery bags for each batch of PL prior to stocking. After qualitative and gross examination, the PL samples were then put in chilled water for 1 to 3 h and transferred to a field laboratory. Each PL batch was then divided into 8 sub-samples of 50 PL each and a separate sub-sample of 100 PL. All the sub-samples were fixed in absolute methanol in separate cryovials and brought to the laboratory for PCR analysis. The overall protocol is illustrated in Fig. 3 (see 'Results' section).

Sample preparation and PCR analysis. Each sub-sample of 50 PL was considered as a separate sample for the PCR analysis and was processed separately using disposable equipment to avoid cross-contamination. Prior to DNA extraction, the anterior portion of all the PL was removed. DNA extraction was performed by an alkaline cell lysis method (Kiatpathomchai et al. 2001). Briefly, each sample of 50 PL was drained and thoroughly homogenised with 3 ml of DNA extraction solution (0.025% sodium dodecyle sulfate and 0.05 N NaOH) in a sterile mortar. Approximately 500 µl of the tissue homogenate was then transferred to a 1.5 ml micro-centrifuge tube, boiled for 5 min, and immediately placed at 4°C until use.

PCR was performed using the method described by Lo et al. (1996b) using primers 146F1/146R1 for the preliminary amplification and 146F2/146R2 for the second nested amplification. All the PCRs were carried out in 100 µl of reaction mixture containing 3 µl of template DNA (approximately 100 ng), 10 µl of 10× reaction buffer (750 mM Tris/HCl pH: 9.0, 200 mM $(NH_4)_2SO_4$, 0.1% Tween-20, 15 mM $MgCl_2$), 100 pmol of each primer, 2.5 units of *Taq* DNA polymerase

(Hybaid-AGS Gold™, UK), 200 µM of each dNTP and sterile double-distilled de-ionized water to make up the final volume. Amplification was performed in a thermocycler (PCR Express) using the following protocol: 1 cycle at 94ºC for 4 min, 55°C for 1 min and 72°C for 2 min; 39 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, followed by a final extension for 5 min at 72°C. Electrophoresis was performed by loading 10 µl of the amplified product onto 1.5% agarose gel with 1× TBE (Trizma, boric acid, EDTA) buffer. The gel was stained using ethidium bromide solution (1 µg ml^{-1}) for 30 min, and the bands were visualised by UV transillumination.

Samples that tested negative by 1-step PCR were subjected to the 2-step nested PCR using 10 µl of the 1-step amplified product as a DNA template.

PCR controls. All PCRs were conducted according to PCR laboratory set-up specifications (Kwok & Higuchi 1989, Dieffenbach et al. 1995) to avoid contamination. The steps in the PCR such as sample preparation, pre-PCR reagent preparation, post-PCR, and 2-step PCR were physically separated and performed in different rooms. DNA samples and PCR reagents were handled with positive displacement pipettes and tips with filter barriers to prevent carry-over of aerosols. Separate sets of equipment were used for sample preparation, pre-amplification, post-amplification and 2-step PCR work. Disposable gloves were worn at all times and were changed frequently at each step, and separate laboratory coats were worn for the pre-PCR and post-PCR areas. Utmost care was taken to prevent sample-to-sample contamination. A negative control was always included in each PCR run; this contained all the PCR reagents except the template DNA and was processed and loaded immediately after the positive control at each step. This allowed a check on any carry-over or cross-contamination.

PCR sensitivity assay. The limit of detection of the present PCR assay was tested by examining 10-fold dilutions of DNA extracted from individual PL that were 1-step and 2-step-positive by PCR. The positive extract was diluted in DNA extracted from uninfected individual PL that were negative by 2-step PCR. These uninfected individual PL were obtained from a 2-step PCR-negative batch. The 10^{-6} dilution had an equivalent of approximately 100 fg of total DNA extract from infected PL, whereas the 10^{-9} dilution corresponded to approximately 100 ag. Three individual PL-positive by 1-step PCR alone, and 4 individual PL-positive only by 2-step PCR were each examined for the limit of detection.

Prevalence estimation. The prevalence of infection in batches was determined by testing a maximum of 300 individuals (6 sub-samples each of 50 PL) by PCR from each of the 73 batches of PL. This allowed the detection of WSSV with 95% confidence if it were present in at least 1% of the population in each batch of PL (Canon & Roe 1982). Initially 200 PL (4 sub-samples each of 50 PL) from each batch were tested and the results recorded. The batches that were negative at this stage were then subjected to further testing until a positive result was obtained or 300 PL had been tested.

A batch was considered positive or infected when at least 1 sub-sample tested positive either by 1-step or 2-step PCR. A batch was considered 1-step positive when at least 1 sub-sample of that batch tested positive by 1-step PCR alone, and a batch was considered 2-step-positive if the sub-sample(s) tested positive only after 2-step nested PCR.

An additional 10 sub-samples, each containing 5 PL, were tested from each of the 1-step-positive batches. In order to assess the prevalence of infection in individual PL, 10 individual PL each from 1-step-positive batches, 2-step-positive batches and a 2-step-negative batch were also tested. These individual PL and sub-samples of 5 PL were taken from sub-samples of the remaining 100 PL from the respective batches.

RESULTS

Limit of detection of PCR assay

The results of the limit of detection assay (Table 1) showed that the 2-step nested PCR could detect WSSV DNA up to a dilution of $1:10^6$ for a 1-step-positive sample whereas it could detect up to a dilution of $1:10^4$ for a 2-step-positive sample. The individual 2-step-positive PL used to carry out the limit of detection test were derived from 1-step-positive batches (Batches 1 and 3) since we could not find any individual PCR-positive PL

Table 1. *Penaeus monodon*. Limit of detection of the PCR assay for different levels of infected postlarvae (PL) from 1-step-positive batches. nd: not detectable

Level of infection	1-step PCR	2-step PCR
1-step-positive PL (heavily infected)		
PL from Batch 1	$1:10^4$	$1:10^6$
PL from Batch 2	$1:10^1$	$1:10^5$
PL from Batch 3	$1:10^2$	$1:10^6$
Average	$1:10^{2.3}$	$1:10^{5.7}$
2-step-positive PL (lightly infected)		
PL from Batch 1	nd	$1:10^4$
PL from Batch 1	nd	$1:10^3$
PL from Batch 3	nd	$1:10^2$
PL from Batch 3	nd	$1:10^2$
Average	nd	$1:10^{2.75}$

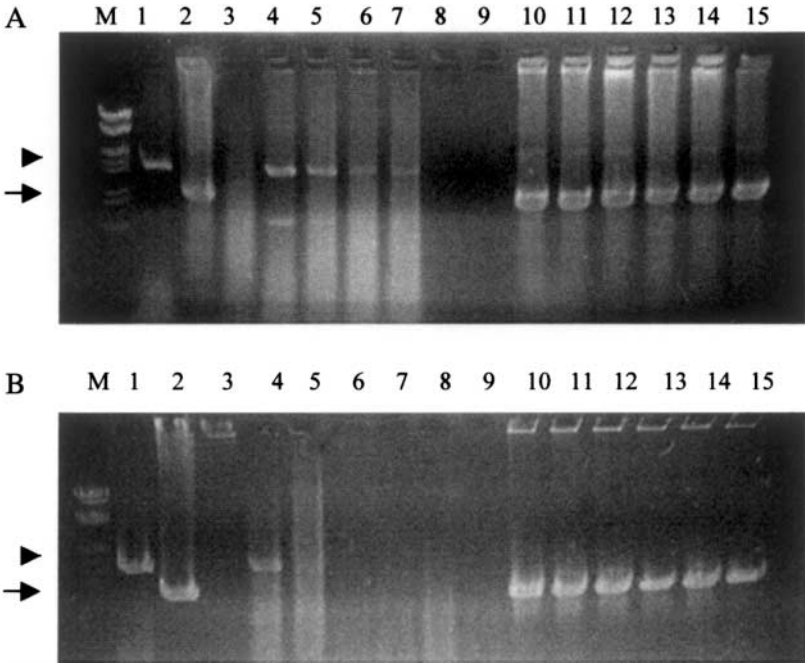

Fig. 1. *Penaeus monodon*. Limit of detection of 1-step and nested PCR for 2 different (A and B) 1-step-positive postlarvae (PL). M: DNA size marker (λDNA double digest with *Eco*RI and *Hin*dIII); Lane 1: 1-step-positive control; Lane 2: 2-step-positive control; Lane 3: negative control; Lanes 4 to 9: 1-step PCR product of 10^{-1} to 10^{-6} dilution of DNA from infected PL; Lanes 10 to 15: nested PCR product of same. Note 1-step PCR product of 1447 bp (arrowheads) and 2-step PCR product of 941 bp (arrows)

Table 2. *Penaeus monodon*. Proportion of postlarvae (PL) batches testing positive for WSSV by PCR (with a sample size to detect a minimum of 1% prevalence). Total N: total number of PL batches tested

Total N	1-step PCR-positive batches	Batches tested positive only by 2-step PCR	Total positive batches	Total batches tested negative
73	3 (4.11%)	33 (45.21%)	36 (49.32%)	37 (50.68%)

(out of 30 PL tested) from the group of 2-step-positive PL batches. Fig. 1 shows the limit of detection result of both 1-step and 2-step PCR for 2 different 1-step-positive PL and Fig. 2 shows the limit of detection result of the 2-step PCR for 2 different 2-step-positive PL. Here, a 1-step-positive sample refers to individual PL tested positive by 1-step PCR alone and a 2-step-positive sample refers to individual PL testing positive only after 2-step PCR.

Prevalence of WSSV in PL population

Out of 73 batches of PL tested, a total of 36 were positive for WSSV either by 1-step or 2-step nested PCR (Table 2). This suggested that approximately 49% (36 of 73; 95% confidence interval, CI = 37.4 to 61.3%) of the PL batches stocked around Kundapur estuary carried WSSV. Amongst the tested batches, only 3 (3 of 73 = 4%) were positive by 1-step PCR. The remaining 37 batches were negative (37 of 73 = 51%) by nested PCR. The details of the PCR results of 50 PL subsamples from the positive batches are given in Table 3 and the approaches with which sub-samples were obtained from various batches for PCR analysis is displayed on the flow chart in Fig. 3.

Examination of the distribution of 2-step-positive results of the sub-samples of 50 PL taken from each of the

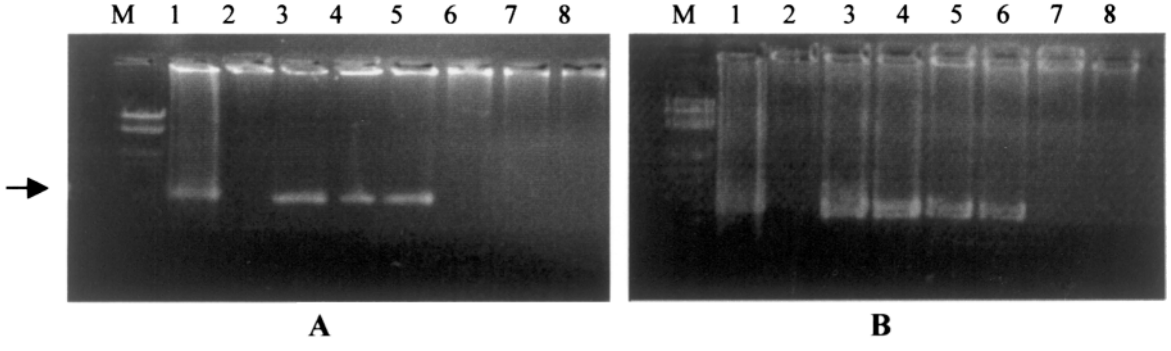

Fig. 2. *Penaeus monodon*. Limit of detection of the nested PCR for 2 different (A and B) 2-step-positive postlarvae (PL). M: DNA size marker (λDNA double digest with *Eco*RI and *Hin*dIII); Lane 1: 2-step-positive control; Lane 2: negative control; Lanes 3 to 8: nested PCR product of 10^{-1} to 10^{-6} dilution of DNA from infected PL. Note 2-step PCR product of 941 bp (arrow)

2-step-positive batches showed that in the majority of batches (25 of 73 = 34%) only 1 sub-sample (1/4, 1/5, 1/6) was positive out of 4, 5 or 6 tested. In 7% (5 of 73) batches, 2 sub-samples tested positive (2/4) and in 4% (3 of 73), 3 sub-samples were positive (3/4). None of the batches was positive in as many as 4 sub-samples.

In the 1-step-positive batches, all 4 sub-samples of 50 PL from each batch were positive. In the first 2 batches (Batches 1 and 2), all the sub-samples were 1-step-positive, whereas in the third batch (Batch 3), 2 sub-samples were 1-step-positive and the other 2 were 2-step-positive. When the additional sub-samples of 5 PL were tested from the 1-step-positive batches, they were all found to be positive by 1-step or 2-step PCR. There was, however, a significant difference (Fisher's exact test, $p < 0.05$) in the numbers positive by 1- and 2-step PCR amongst different batches (Table 4).

Prevalence of WSSV in individual PL from 1-step and 2-step WSSV-positive batches

Ten individual PL randomly selected from all three 1-step-positive batches, three 2-step-positive batches and one 2-step negative batch were analysed, and the results are shown in Table 5.

In all the 1-step PCR-positive batches 10 of 10 (100%) of the PL were positive, and they differed significantly amongst batches (Fisher's exact test, $p < 0.05$) in the proportion of 1-step-positive PL. In the case of 2-step-positive batches (3/4, 2/4, 1/4) and the 2-step negative batch (0/6), not a single PL was found to be positive, suggesting a low prevalence.

DISCUSSION

The present study investigated the prevalence of WSSV in *Penaeus monodon* PL at the time of stocking in Indian shrimp farms. The point prevalence of WSSV-infected PL batches in the entire population of PL batches stocked in the Kundapur estuary between September 1999 and January 2000 was 49%, with a 95% CI of 37.4 to 61.3%. A sample size of 300 PL from each batch was used, since detecting a prevalence of ≥1% or above in a population of ≥10 000 individuals requires a sample of 297 PL (Canon & Roe 1982). Thus, the prevalence of 49% refers to the probability of a sample of PL batches testing positive by 2-step PCR at a sampling level selected to detect prevalence of WSSV infection of 1% or more in the population of PL within a batch.

The limit of detection of the PCR assay was shown to be much lower than the equivalent of 1 PL in 50. In fact, at $1{:}10^5$ to $1{:}10^6$ dilutions, WSSV DNA could be detected by the nested PCR. At this dilution the original template DNA from the infected tissue corresponded to 1 pg to 10 fg. Lo et al. (1996b) also detected WSSV DNA at up to 10^{-9} dilution (1 fg of template DNA) from deproteinized DNA extract from muscle tissue of an infected adult *Penaeus monodon* shrimp.

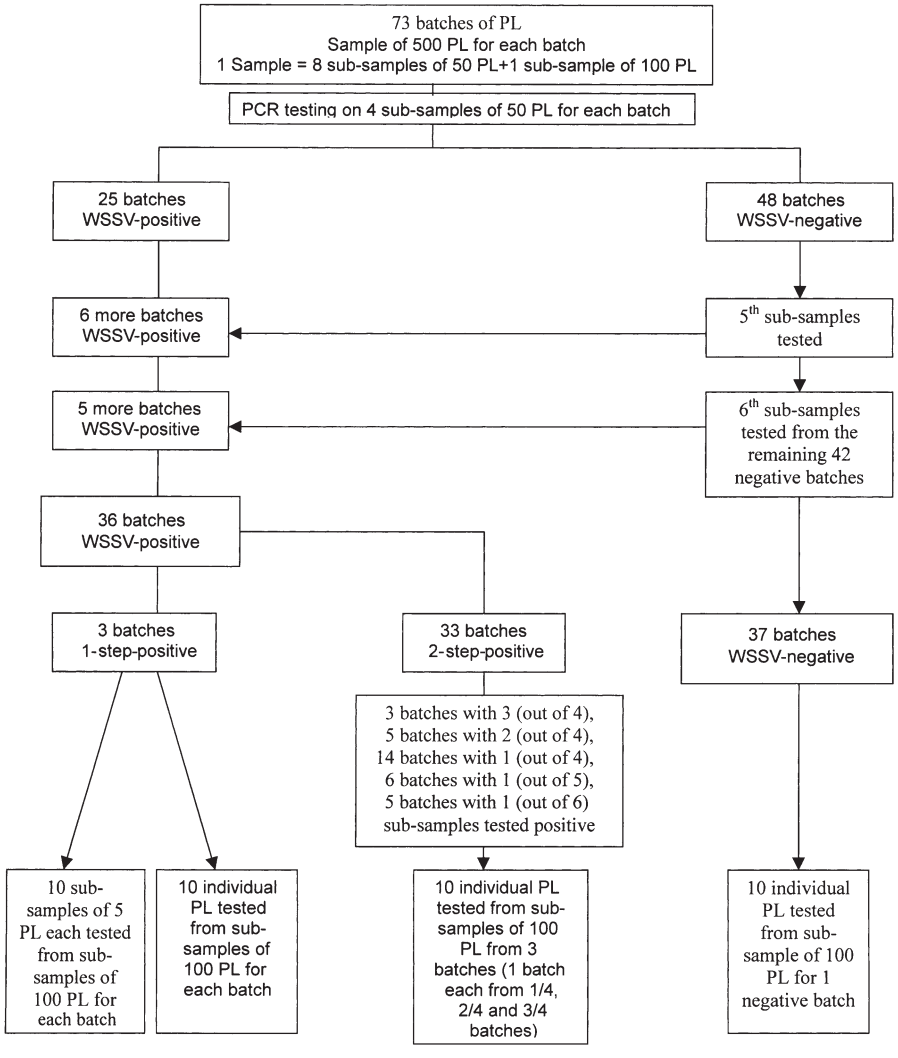

Fig. 3. *Penaeus monodon*. Flow diagram showing sampling scheme and results for sub-sample processing of PCR tests

Table 3. *Penaeus monodon*. PCR results of sub-samples of 50 postlarvae (PL) from WSSV-positive batches. ++: 1-step-positive, +: 2-step-positive, NA: not analysed

PL batch no.	PCR results for sub-samples 1st	2nd	3rd	4th	5th	6th	Proportion of WSSV-positive sub-sample
1	++	++	++	++	NA	NA	4 of 4
2	++	++	++	++	NA	NA	4 of 4
3	+	+	++	++	NA	NA	4 of 4
4	+	+	+	–	NA	NA	3 of 4
5	–	+	+	+	NA	NA	3 of 4
6	–	+	+	+	NA	NA	3 of 4
7	+	+	–	–	NA	NA	2 of 4
8	–	+	–	+	NA	NA	2 of 4
9	+	–	+	–	NA	NA	2 of 4
10	+	+	–	–	NA	NA	2 of 4
11	–	–	+	+	NA	NA	2 of 4
12	–	+	–	–	NA	NA	1 of 4
13	–	–	+	–	NA	NA	1 of 4
14	–	+	–	–	NA	NA	1 of 4
15	–	–	–	+	NA	NA	1 of 4
16	–	+	–	–	NA	NA	1 of 4
17	+	–	–	–	NA	NA	1 of 4
18	–	–	+	–	NA	NA	1 of 4
19	–	–	+	–	NA	NA	1 of 4
20	–	–	+	–	NA	NA	1 of 4
21	–	+	–	–	NA	NA	1 of 4
22	–	–	–	+	NA	NA	1 of 4
23	–	+	–	–	NA	NA	1 of 4
24	–	–	–	+	NA	NA	1 of 4
25	+	–	–	–	NA	NA	1 of 4
26	–	–	–	–	+	NA	1 of 5
27	–	–	–	–	+	NA	1 of 5
28	–	–	–	–	+	NA	1 of 5
29	–	–	–	–	+	NA	1 of 5
30	–	–	–	–	+	NA	1 of 5
31	–	–	–	–	+	NA	1 of 5
32	–	–	–	–	–	+	1 of 6
33	–	–	–	–	–	+	1 of 6
34	–	–	–	–	–	+	1 of 6
35	–	–	–	–	–	+	1 of 6
36	–	–	–	–	–	+	1 of 6

Table 4. *Penaeus monodon*. PCR results of the sub-samples (5 postlarvae, PL, each) tested from sub-samples of 100 PL from the 1-step-positive batches. Total N: total number of sub-samples tested

1-step-positive batches	Total N	No. of 1-step-positive sub-samples	No. of sub-samples positive only by 2-step PCR
Batch 1	10	1	9
Batch 2	10	10	0
Batch 3	10	2	8

Table 5. *Penaeus monodon*. PCR results of the individual postlarvae (PL) tested from sub-samples of 100 PL from different batches. N: number of PL tested

PL batches	N	No. of 1-step-positive PL	No. of PL positive only by 2-step PCR	No. of negative PL
2-step-negative batch	10	0	0	10
2-step-positive batch				
3/4	10	0	0	10
2/4	10	0	0	10
1/4	10	0	0	10
1-step-positive batch				
Batch 1	10	3	7	0
Batch 2	10	10	0	0
Batch 3	10	1	9	0

The limit of detection may vary, depending on the initial quantity of target DNA in the original sample or the template DNA. However, quantification was not performed in either study to estimate the concentration of WSSV DNA in the samples tested. The assay demonstrated that 2-step nested PCR was 10^3 to 10^4 times more sensitive in detecting WSSV DNA than the 1-step PCR alone. This supports the results of nested PCR sensitivity for WSSV detection as reported by Lo et al. (1996b). This would suggest that samples which are 1-step PCR-positive must have at least 10^3 to 10^4 times more viral DNA than samples revealed as positive only by 2-step nested PCR. Based on this, we scored the PL batches and the individual PL as heavily infected (1-step-positive) and lightly infected (2-step-positive).

In our limit of detection assay, we used DNA extracted from uninfected shrimp (2-step negative) as the diluting medium instead of the dilution buffers or double-distilled deionised water, since PCR sensitivity may tend to be lower in the presence of excess shrimp genomic DNA. This provided a more realistic estimate of the sensitivity of the nested PCR procedure in a routine screening situation where only 1 shrimp may be infected out of several in a sample. The present nested PCR technique was able to detect a minimum equivalent to 1 heavily infected PL (1-step-positive) in 1 million. Similarly, for the lightly infected PL (2-step-positive), the minimum and maximum limits of detection were the equivalent of 1 in 10 000 and 100 respectively. Although the sensitivity of the nested PCR was sufficient to detect the presence of infected PL in large sample sizes, it was often more convenient to conduct PCR analysis on sub-samples of 30 to 50 PL for easier handling and processing.

Variations in the limit of detection from different individuals could be attributable to the level of viral load in the initial sample. Thus, by a dilution technique with PCR, an indirect quantal assay can be performed to asses the infection level/viral load in individuals or groups. However,

the possibility of false negatives cannot be ruled out where the viral load or the prevalence is extremely low. A PCR-negative status means that the WSSV target DNA is below the detectable limit in the template DNA added to the reaction mixture, and does not guarantee WSSV-free status. Further, different PCR assays would offer different levels of sensitivity, depending on the preservation method, DNA extraction method, template concentration and the size of amplicons (Sambrook et al. 1989, Cha & Thilly 1995). For instance, a sample that tests positive at first amplification by 1 diagnostic PCR protocol may test positive only after re-amplification by a less sensitive protocol. Similarly, a 2-step-positive sample tested by a highly sensitive protocol may test (falsely) negative by a less sensitive protocol. DNA extraction methods such as phenol-chloroform extraction or hot phenol extraction can be superior in terms of recovery of DNA and the stability of DNA, but they are time-consuming and expensive and this negates the benefit of rapid PCR or may not be feasible for large numbers of samples. Such extensive DNA extraction procedures must also be weighed against the increasing risk of sample contamination and loss of DNA at each manipulation step. In a more recent study, the PCR sensitivity was reported to be sufficient when using the template DNA prepared from WSSV-infected samples by the alkaline extraction method (Kiatpathomchai et al. 2001). In the present investigation, the combination of alkaline DNA extraction and 2-step nested PCR (Lo et al. 1996b) proved to be rapid and sensitive.

The most widely used fixative for PCR analysis is ethanol, but for our purpose we substituted methanol because of the non-availability of ethanol in the large quantity required for this study. The samples were stored in absolute methanol for a period of 3 to 6 mo prior to PCR analysis. The duration for which the samples are maintained in the fixative is also reported to be critical, as the type of fixative and certain fixatives also affect the ability of PCR to effectively amplify a larger target sequence (Greer et al. 1995). The possible impact of methanol towards lowering the recovery of intact DNA and the efficiency of subsequent PCR amplification is not known. However, from our limit of detection result, it is evident that there may be little or no impact on WSSV detection by the present method arising from such effects.

A significant aspect of the results was the variability in proportion of sub-samples that were positive in different 2-step-positive batches, especially when there was low levels of infection. The majority of the 2-step-positive batches had only 1 positive sub-sample out of 4, 5 or 6 tested and in no case were all the sub-samples positive. This clearly shows that when a batch is only 2-step-positive, the prevalence of infected individuals in the batch might be very low. This is clear from the fact that the other 150, 200 or 250 shrimp in such PL batches gave negative test results (since the limit of detection was $<1/50$). Testing 150 individuals allows 95% confidence in detecting up to a minimum of 2% prevalence. Therefore, it appears that these batches had a prevalence below 2%. These results suggest that batches with low WSSV prevalence might yield some samples that test positive and others that test negative even when tested by the same PCR protocol; this could occur when very few, unevenly distributed, infected PL are present in a population. In commercial screening practices, usually 25 to 50 PL are tested for assessing the infection status of PL batches, which may usually number up to 100 000 or more PL, representing the progeny of several spawners. This may result in a high probability of sampling error and consequent falsely negative results. An earlier report (Withyachumnarnkul 1999) also documented that such false negatives are likely, and it was suggested that 150 individuals be homogenised instead of 50. Essentially, the results of our study confirm that variation in the results of 2-step PCR using individual samples of 50 PL as commonly used in commercial testing carries a significant risk of obtaining a falsely negative result for the population being tested. Testing 50 or 150 individuals from a population of infinite size allows 95% confidence of detecting 5 and 2% prevalence respectively. Assaying a minimum of 300 PL from a population either pooled or in sub-samples of 50 PL would further reduce the probability of falsely negative results. Such a procedure is essential in epidemiological studies concerning risk-factor analysis, in which a very low prevalence of infection in a batch has to be detected.

In this study we found that all sub-samples of 5 PL and individual PL tested from the 100 PL groups of the three 1-step-positive batches were positive either by 1-step or 2-step nested PCR. In contrast, all the sub-samples of individual PL taken from the 2-step-positive batches tested negative. These results further substantiate the higher prevalence of WSSV in 1-step-positive batches and lower prevalence in 2-step-positive batches. Similarly it was found that in some of the 1-step-positive batches, the proportion of 1-step-positive PL (heavily infected) might be very high (as in the case of Batch 2), whereas in others there might be a very low number of heavily infected PL.

A critical aspect of this study was the diagnostic test used to determine infection status. PCR detects only a fragment of DNA. Whether the virus is viable or infectious cannot be known, and a non-viable virus present on the sample surface can also contribute to a PCR-positive result. Due to lack of a continuous shrimp cell line, PCR has been the sole and routine diagnostic tool in detecting WSSV infection in latent carriers or lightly

infected shrimp. On the other hand, because of the exquisite sensitivity of 2-step nested PCR, passive contamination is a major concern. The sub-samples of 5 PL each and the individual PL were taken from the PL 100 sub-samples fixed in 1 cryovial for the respective batches. The possibility of surface contamination from one PL to the other cannot be ruled out. However, these PL were fixed intact and live and there was no damage to the tissue or release of potentially infectious tissue fluids or haemolymph that could cause contamination. Hence, the possibility of such passive contamination is unlikely. Moreover, all these sub-samples and individual PL were processed using separate equipment to further reduce any cross-contamination.

The present study examined the PL at the time of stocking and not from hatcheries. This provided a better assessment of the level of infection introduced to the pond with the PL. Transport stress could have caused prepatent infections to become transitional or patent. Earlier reports have suggested that development from prepatent to transition or patent stage of infection occurred due to stress caused by pereiopod excision (Peng et al. 1998) and spawning stress (Lo et al. 1997), bringing the virus level in the sample to a detectable limit of 1-step PCR from 2-step PCR, thus improving the chance of detection. Transport stress has also been reported to affect the health quality of PL (Chanratchakool et al. 1998). However, no data on the infection status of the PL batches from the hatcheries was collected, and therefore it is not possible to comment on the effect of transportation. During the sample handling, the PL were placed in chilled water for 1 to 3 h prior to fixation, which would also stress them. The possibility of enhancement of infection status and thereby an apparent increase in WSSV prevalence within batches during sample handling cannot be ruled out.

This investigation looked into the prevalence status of WSSV in various discrete PL populations at the time of stocking in a major artisanal shrimp farming area on the west coast of India. Previously, there have been very few studies on WSSV prevalence in PL. In an earlier study, while monitoring WSSV in Ecuador (Calderon et al. 2000), the prevalence in hatchery tanks varied from 15 to 92% in different provinces, with sample sizes of 150 PL per batch. In another study carried out in India along the west coast (Otta et al. 1999), 5% of the PL batches tested were positive by 1-step PCR, whereas 48% were positive by nested PCR with a sample size of 20 to 30 PL per hatchery. In this study we found that 49% of the PL batches stocked in the enrolled ponds were positive for WSSV, of which only 4% were positive by 1-step PCR. This, however, is an estimation of point prevalence and there could be temporal and spatial variations. These results substantiate the persistence of WSSV in *Peneaus monodon* PL populations in India. In recent years the prevalence and the viral load entry along with PL batches have been suggested to play a role in WSS outbreaks (Lo et al. 1998). The estimation of prevalence of infection within batches is difficult, and this study represents an attempt to do so. Evaluation of a reliable protocol to quantify the viral load and prevalence of infection within batches would be useful in epidemiological studies.

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