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Detection and Prevalence of white spot syndrome disease (WSSV) in shrimp farms

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ABSTRACT

White spot syndrome is a viral infection of *Penaeus monodon* shrimp. It is highly lethal, contagious and killing shrimps and doesn't have a specific anti-viral treatment for this infection. This important infection affects on shrimp farms in Sri Lanka and leads to the loss of economy. Most of shrimp products are export oriented. Therefore this study aimed to determine the factors, detection and prevalence of WSSV in shrimp farms, Sri Lanka. The prevalence rate of WSSV among the collected samples (n = 100) is 58.05% (2011), 59.06% (2012) in Sri Lanka respectively. Therefore the infection and resistance of virus has increased among the shrimp population annually. Further studies need to be identified the WSSV aggressive viral strains and prevention strategies to evade from this sever infection in shrimp farming.

***Keywords*:** WSSV; DNA genome; OIE; Penaeid shrimps; Nested PCR

1. INTRODUCTION

White spot disease is a contagious, lethal viral disease of *Penaeid* prawn. It is an enveloped, rod-shaped virus containing a double-stranded DNA genome. WSSV is classified

as a member of the Whispoviridae (Flegel, 2006; Jang et al., 2009; Meng et al., 2009). WSSV was first reported from both Taiwan and the People's Republic of China in 1992 (Jang et al., 2009).Apparently, it has spread throughout East, Southeast and South Asia, North, South and Central America (Jang et al., 2009). White spot syndrome is a disease listed by the OIE (Council Directive 2006/88/EC on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals).

White spot syndrome virus has been detected in a wide range of decapod and non-decapod crustacean hosts from natural environments (Jang et al. 2009). In addition, all decapod crustaceans from marine and brackish or freshwater sources that have been subjected to experimental infection trials have been successfully infected (Wang et al. 1999a).The environmental stress factor is a high alkalinity (Wang et al. 1999a).

Penaeid species infected with WSSV can culture and display obvious white spots or patches of 0.5–3.0 mm in diameter embedded in the exoskeleton (Lo, Leu, Ho, Chen, Peng, Chen, Chou, Yeh, Huang, Chou, Wang & Kou 1996a). The exact mechanism of white spot formation is not known. It is possible that a WSSV infection may induce the dysfunction of the integument resulting in the accumulation of calcium salts within the cuticle and giving rise to white spots (Wang et al. 1999a). The other signs of disease include a reddish discolouration of body and appendages because of the expansion of chromatophores (Nadala et al. 1998), reduction in feed uptake (Chou et al. 1995), preening and response to stimulus (Wongteerasupaya et al. 1995), loose cuticle (Wang & Kou 1996b), swelling of branchiostegites because of accumulation of fluid (Karunasagar & Karunasagar 1999), enlargement and yellowish discolouration of the hepatopancreas (Sahul-Hameed et al. 1998) and thinning and delayed clotting of haemolymph (Wang et al. 2000). The presence of white spots does not always mean that the condition is terminal. According to some literature, there are four main strains have been identified. However, if the shrimp also appear lethargic, if their colour changes to pink or reddish-brown, if they gather around the edges of ponds or tanks at the water surface, or if there is a rapid reduction in food consumption, then a very high mortality rate in the shrimp population can be expected within a few hours to a few days of the onset of these signs.

The molecular detection kit totally made locally for detecting WSSV in shrimps, but it is very cost effective. A viral DNA extraction kit and nested PCR technique was developed for simultaneous detection and severity grading of white spot syndrome virus (WSSV) infections in t shrimps. It helps to distinguish susceptibility to infection, because the technique only detects the presence of the elements of the viral genome. Whether the viral genome is present intracellularly or on the surface or in the gut content of the animal needs to be confirmed by histology, in situ hybridization, immunohistochemistry or transmission electron microscopy. All farmed *penaeid* shrimp species from late post-larvae to juvenile and adult stages are highly susceptible to infection. Clinical signs appear on-farm after 14–40 days of stocking. The characteristic white spots are rarely present, particularly in Penaeus vannamei. Mortalities may reach 100% within 5 days after the onset of the disease. Survivors may carry the virus for life and may pass the virus to their progeny (Lo et al., 1996, 1997).

There are several pathways of transmitting of disease, likely vertical transmission and horizontally transmission. Egg-associated transmission is suspected vertical transmission. Horizontal transmission can be introduced by the consumption of infected tissue and waterborne routes (Lo et al., 1997; Chou et al., 1998; Lo and Kou, 1998). Transmission of infection can occur among apparently healthy animals. As well dead animals can be a source

of infection (Lo et al., 1997; Chou et al., 1998; Lo and Kou, 1998). Tissue tropism analysis from both experimentally infected shrimp and wild-captured brooders shows that tissues originating from the ectoderm and mesoderm, especially the cuticular epithelium and subcuticular connective tissues. The main target tissues for WSSV is the pleopods, gills, haemolymph (Lo et al., 1997). In addition, the effects of the disease in farmed shrimp, the pathogen has been detected in wild crustaceans (Flegel, 2006; Jang et al., 2009; Meng et al., 2009).

Genome and classification

The WSSV genome is a circular, ds DNA molecule with an A+T content of 59% homogeneously distributed. The genome size varies according to the viral isolate (Chen,Wang,Huang,Peng, Chen, Lin, Chen, Dai, Yu & Wang 2002b).

2. MATERIAL AND METHODS

Field Diagnosis

The main purpose of diagnosis of WSSV is to confirm the occurring of an outbreak throughout the hateries. It helps to ensure that brood stock shrimp and shrimp larvae are pathogen-free for disease surveillance (Flegel et al 1993). Clinical signs White spots embedded within the exoskeleton are the most commonly observed clinical sign. In most shrimp, these spots range from barely visible to 3 mm in diameter.

Sampling

Samples (n = 100) were collected from several hatcheries in Ambakadawilla, Kalpitiya and several rurel areas in Negombo lagoon annually. Samples were labeled and stored under -70c .Desect pleopods and gills and store separately.

Fig. 1. Gills separation

Clinical Pathology

Histology

H&E staining reveals intranuclear inclusion bodies as prominent eosinophilic to pale basophilic in hypertrophied nuclei. Most commonly seen in the cuticular epithelial cells and connective tissue cells, and, less frequently, the antennal gland epithelium, lymphoid organ sheath cells, haematopoietic cells and fixed phagocytes of the heart. Feulgen staining reveals the intranuclear inclusion bodies to be Feulgen positive. Intranuclear occlusion bodies are absent

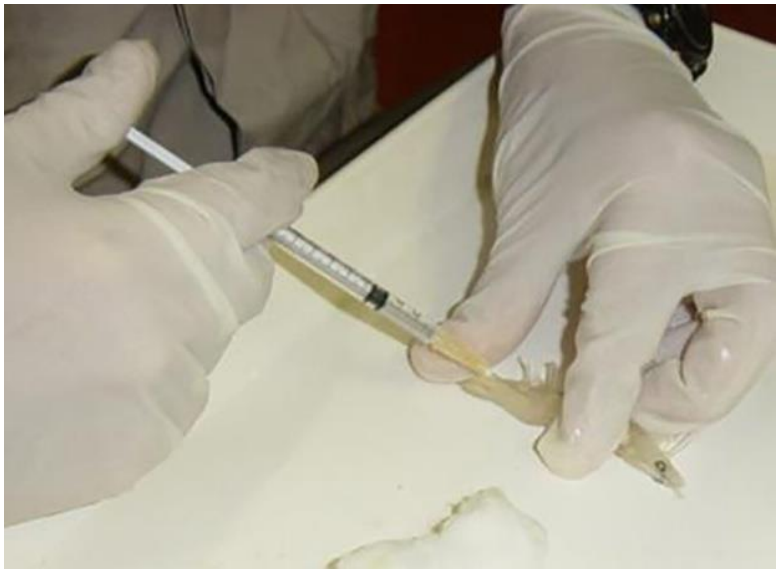


Fig. 2. Injecting stain

DNA Extraction and Amplification

The protocol describs for all situations where WSSV diagnosis is required (Lo et al. 2004). A positive result in the first step of this standard protocol implies a serious WSSV infection, whereas, when an only positive result is obtained in the second amplification step and a latent or carrier-state infection is indicated as well. PCR commercial kits are available for WSSV diagnosis.

DNA extraction

According to Promega 2000 KIT mannual

Collect 100-200 mg shrimp tissue (pleopod of live juvenile to subadult shrimp, postlarvae 11 upwards [PL11 up] with removed heads, or whole PL10, or use 100 µl haemolymph) in a 1.5 ml microfuge tube with 600 µl lysis solution (100 mM NaCl, 10 mM Tris/HCl, pH 8, 25 mM EDTA [ethylene diamine tetra-acetic acid], 0.5% SLS [sodium N-laurylsarcosinate] or 2% SDS [sodium dodecyl sulphate], and add 0.5 mg ml–1 proteinase K added just before use). Using a disposable stick, homogenise the tissue in the tube thoroughly. After homogenisation, incubate at 65 °C for 1 hour. Add 5 M NaCl to a final concentration of

0.7 M. Next, slowly add 1/10 volume of N-cetyl-N,N,N-trimethylammonium bromide (CTAB)/NaCl solution (10% CTAB in 0.7 M NaCl) and mix thoroughly. Incubate at 65°C for 10 minutes, and then, at room temperature, add an equal volume of chloroform/isoamyl alcohol (24:1) and mix gently. Centrifuge at 13,000 g for 5 minutes and then transfer the aqueous solution (upper layer) to a fresh 1.5 ml tube and add an equal volume of phenol. Mix gently and centrifuge at 13,000 g for 5 minutes. Collect the upper layer solution and repeat the phenol extraction process once or twice. Transfer the final upper layer to a new tube, mix gently with two volumes of chloroform/isoamyl alcohol (24:1) and centrifuge at 13,000 g for 5 minutes. Transfer the upper layer to a new tube and precipitate DNA by adding two volumes of 95% or absolute ethanol followed by standing at –20 °C for 30 minutes or –80 °C for 15 minutes. Centrifuge at 13,000 g for 30 minutes and discard the ethanol. Wash the DNA pellet with 70% ethanol, dry and resuspend in 100 µl sterilised double-distilled water at 65°C for 15 minutes.Use 1 µl of this DNA solution for one PCR reaction.

The DNA amplification method is following by using nested PCR procedures are well established and provide reliable diagnostic results under the specified conditions. However it helps to ensure that DNA samples are prepared from the recommended organs and that the PCR temperature is accurately applied.Beacuse it is particularly needed to be with accurate annealing temerature, the recommended temperature is 62 °C. To prevent the possibility of false positive results, it is important to adhere to the specified procedures.For diagnosed incidences of WSSV in a new host or in a previously free zone, DNA sequencing should be used to confirm the positive results.

Manual of Diagnostic Tests for Aquatic Animals 2009

First-step PCR reaction

i) Add 1 µl DNA template solution (containing about 0.1-0.3 µg DNA) to a PCR tube containing 100 µl of reaction mixture (10 mM Tris/HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X100, 200 µM of each dNTP, 100 pmol of each primer, 2 units of heat-stable DNA polymerase).

ii) The outer primer sequences are ;
146F1, 5'-ACT-ACT-AAC-TTC-AGC-CTA-TCTAG-3'
146R1, 5'-TAA-TGC-GGG-TGT-AAT-GTT-CTT-ACG-A-3'.

iii) The PCR profile is one cycle of 94 °C for 4 minutes, 55 °C for 1 minute, and 72 °C for 2 minutes, followed by 39 cycles of 94 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 2 minutes and a final 5-minute extension at 72 °C. The WSSV-specific amplicon from this reaction is 1447 bp. The sensitivity is approximately 20,000 copies of a plasmid template.

Second step of the (nested) PCR reaction

This second step is necessary for the detection of WSSV in shrimp at the carrier stage.

i) Add 10 µl of the first-step PCR reaction product to 90 µl of a PCR cocktail with the same composition as above except that it contains the second (inner) primer pair: 146F2 (5'-GTA-ACTGCC-CCT-TCC-ATC-TCC-A-3') and 146R2 (5'-TAC-GGC-AGC-TGC-TGC-ACC-TTG-T-3').

ii) Use the same PCR amplification protocol as above. The WSSV-specific amplicon from this reaction is 941 bp. The overall sensitivity of both steps is approximately 20 copies of a WSSV plasmid template.
iii) To visualise, electrophorese 10 µl PCR reaction products on 1% agarose gels containing ethidium bromide at a concentration of 0.5 µg ml–1.
iv) Decapod-specific primers (143F 5'-TGC-CTT-ATC-AGCTNT-CGA-TTG-TAG-3' and 145R 5'TTC-AGN-TTT-GCA-ACC-ATA-CTT-CCC-3' yielding an 848 bp amplicon.It should be used in control reactions to verify the quality of the extracted DNA and the integrity of the PCR reaction. In the penaeid shrimp.The PCR product generated by this decapod-specific primer pair corresponds to nucleotide sequence 352–1200 of the 18s rRNA . The decapod 18s RNA sequence is highly conserved and produces a similar sized PCR product in almost all decapods.
v) A positive control (WSSV DNA template) and negative controls (no template and shrimp DNA template) should be included in every assay.

DNA sequencing of PCR products

For confirmation of suspected new hosts of WSSV, the DNA fragment amplified from the two-step nested diagnostic PCR should be sequenced. The cloning and sequencing protocols described here are according to (Claydon et al.1998).

i) Excise the DNA fragments selected for further analysis from the agarose gels and purify them using any of the commercially available PCR clean up kits.
ii) Ligate amplicons into vector plasmid and clone the construct.
iii) Use suitable primers to amplify the inserted amplican, and then subject the amplified product to DNA sequencing.
iv) Compare the sequences obtained with available databases using the Basic Local Alignment Search Tool (BLAST) to determine approximate phylogenetic affiliations.

OIE Recommended Techniques for Surveillance and Confirmation

The methods listed in the table below are the OIE recommended techniques for surveillance and confirmation testing:

- Pathogen
- Surveillance (Juveniles and Adults only)
- Confirmatory Techniques
- White Spot Syndrome Virus
- Polymerase Chain Reaction (PCR)
- Histology, Transmission Electron Microscopy (TEM), DNA probes in situ, PCR and Sequencing

Surveillance Testing

Polymerase Chain Reaction (PCR)

The suggested protocol is that described by Lo et al. 1997. It is recommended for all situations where WSSV diagnosis is required. A positive result in the first step of this

standard protocol implies an advanced WSSV infection, when a positive result is obtained in the second amplification step only, a latent or carrier state infection is indicated. Commercial PCR diagnostic kits are available and have been very useful in the standardization and harmonisation of the technique. It is recommended that the most recent OIE Diagnostic Manual be consulted for up-to-date developments in molecular diagnostics for WSD.

Sequencing

For confirmation of suspected WSSV, the DNA fragment amplified from the two-step nested diagnostic PCR should be sequenced. The suggested cloning and sequencing protocols are those described by Claydon et al. (2004). It is acceptable to sequence the PCR amplicon directly. If a positive result is obtained, compare the sequences to available databases using the Basic Local Alignment Search Tool (BLAST) to determine approximate phylogenetic affiliations. If a negative result is obtained the sample should be tested again.

3. RESULTS AND DISCUSSION

Transmission Electron Microscopy (TEM)

WSSV particles can be seen within the intranuclear inclusion bodies of infected cells. Virions are rod-shaped to elliptical, non-occluded and measure between 80 - 120 nm in width and 250 - 380 nm in length.

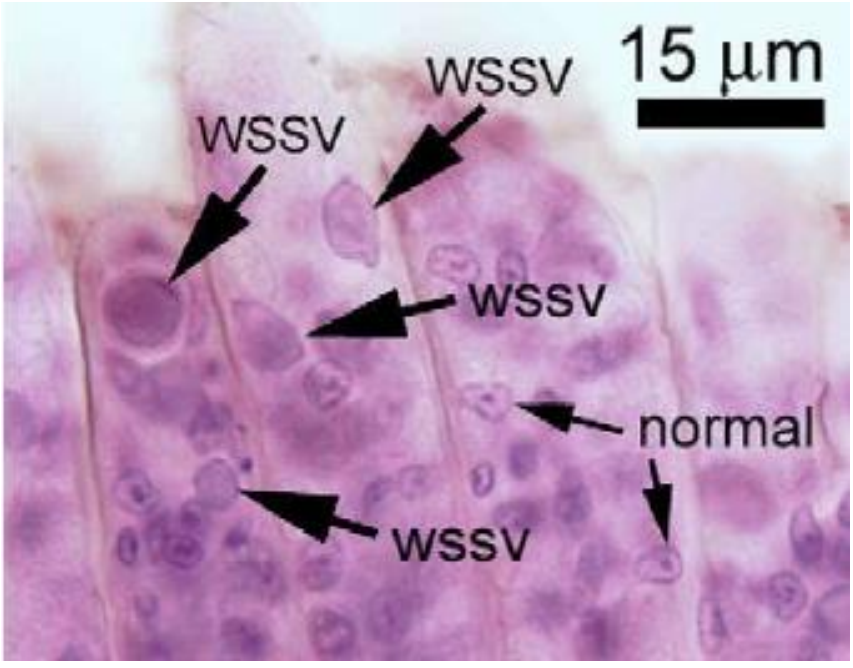


Figure 3. Observation under microscope

Polymerase Chain Reaction (PCR)

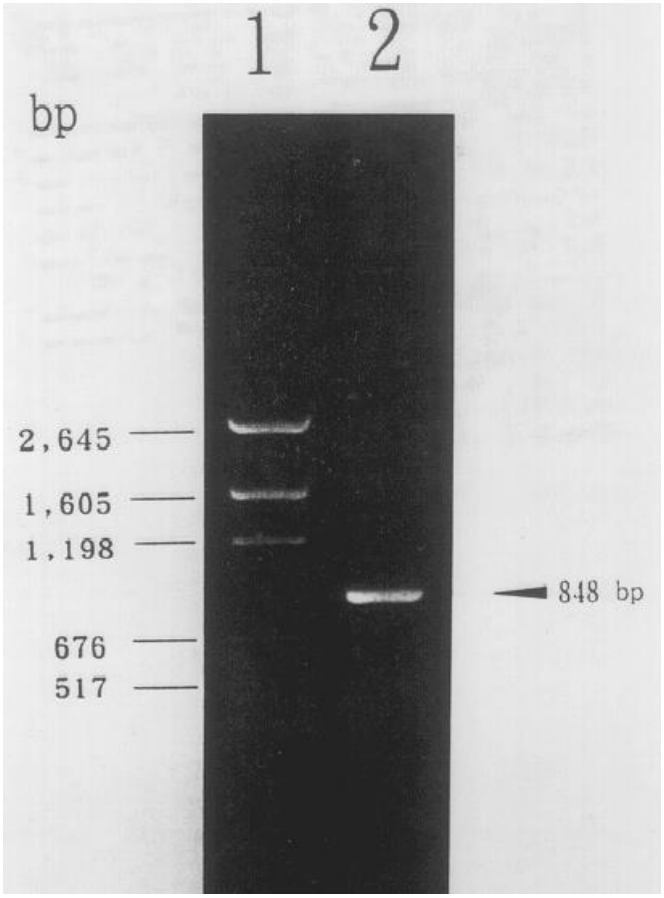
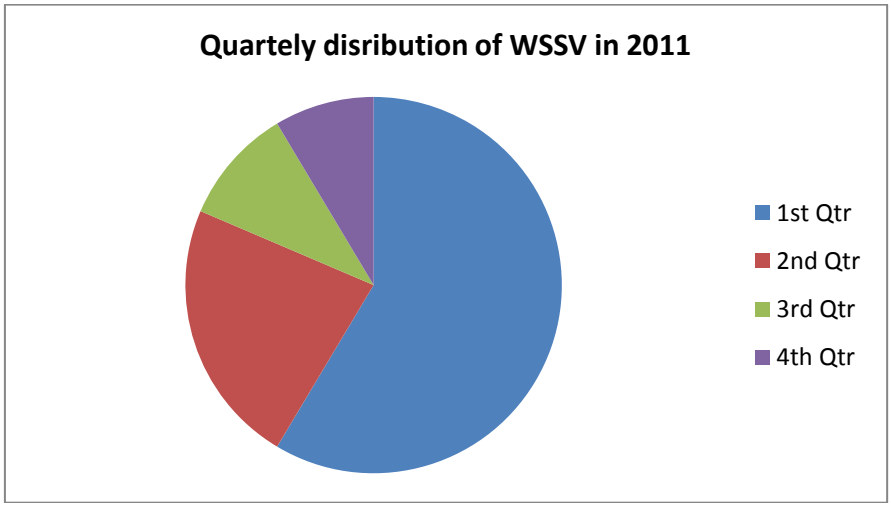


Figure 4. Tissue from a WSSV-infected prawn tested with decapod primers 143F & 145R. A single band of the expected size of 848 bp is visible



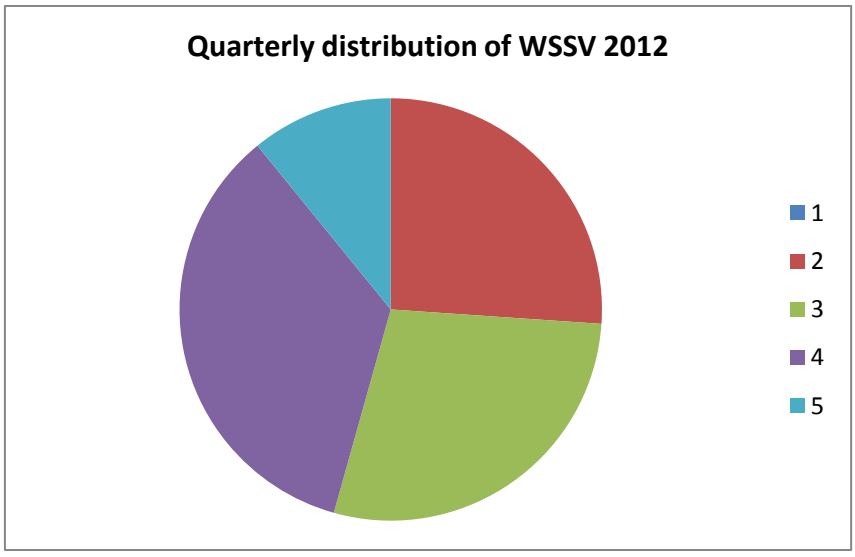


Fig. 5. PCRs for WSSV detection, electrophoresed on a 1% agarose gel.

Lane 1. 100 bp markers. Lower bright band represents 600 bp, and the bands extending up from this band are in 100 bp increments up to 1500 bp;

Lane 2. Tissue from a WSSV-infected prawn sample tested with the WSSV primers 146F1 & 146R1. A single band of the expected size of 1447 bp is visible;

Lane 3. Tissue from a WSSV-infected prawn tested with decapod primers 143F & 145R. A single band of the expected size of 848 bp is visible;

Lane 4. Tissue from an uninfected prawn sample tested with the WSSV primers 146F1 & 146R1. No reaction products are visible in this reaction;

Lane 5. Tissue from an uninfected prawn sample tested with decapod primers 143F & 145R. A single band of the expected size of 848 bp is visible;

Lane 6. Positive Control: a plasmid DNA clone containing the 941 bp nested fragment of WSSV, tested with primers 146F2 & 146R2. A single band of the expected size of 941 bp is visible.

4. DISCUSSION

There are no pathognomonic clinical signs or lesions associated with WSD. White spots on the cuticle are not observed in all cases and when they occur, such spots can also be caused by bacterial infection or environmental conditions. A presumptive diagnosis in clinically affected animals may be made on recognition of characteristic microscopic changes in tissues but a confirmed diagnosis of WSD requires the use of polymerase chain reaction as well as in situ hybridisation or transmission electron microscopy. The OIE lists the species susceptible to WSSV infection as all decapod crustaceans from marine and brackish or freshwater sources. Under the conditions of prawn aquaculture used in Asia, screening of broodstock for the presence of WSSV has allowed selection of broodstock with low level of infection and the post-larvae produced by these animals have a greater chance of surviving the entire grow-out periodMaterial for testing should be collected from live or moribund juvenile or adult prawns as degradation of tissue and viral DNA, proceeds rapidly after death. Whole larvae or post-larvae will also suffice. Cuticular epithelium can be obtained by scraping the membranous epithelium from the carapace, extracting a piece of pleiopod or by dissecting it from the abdominal muscle.

The nested PCR amplifies a 1447 base pair (bp) sequence of viral genomic DNA in the first reaction, using primers 146F1 and 146R1 and an internal fragment of 941 bp in the nested reaction, using primers 146F2 and 146R2 . The nested PCR has an overall sensitivity in the range of 18-180 target molecules for non-degraded DNA. Reaction products are visualised by agarose gel electrophoresis. The method is adapted from the OIE Manual of Diagnostic Tests for Aquatic Animals.Commercial kits are available for the detection of WSSV by PCR, and it may be preferable for some laboratories to use one of these kits, using the manufacturer's instructions.

5. CONCLUSION

The nestd PCR is a highly sensitive method and the slightest contamination of samples with WSSV DNA, or with WSSV amplicons, can cause false positive results. To detect cross contamination, it is essential to include appropriate positive and negative controls in each set of samples. Previously the shrimp were infected with low aggressive strains got killed, but now they are resistant to low aggressive strains.

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