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Development of immunodot blot assay for the detection of white spot syndrome virus infection in shrimps (*Penaeus monodon*)

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Abstract

The VP 28 gene encoding a structural envelope protein of the white spot syndrome virus (WSSV) was cloned into a pET32a(+) expression vector for the production of the recombinant VP28 protein. A purified recombinant protein of 39.9 kDa size was used for polyclonal antibody production in rabbit. Specific immunoreactivity of the rabbit anti rVP28 antiserum to the viral antigen was confirmed by a Western blot. The specificity of this polyclonal anti-rVP28 antiserum to detect the presence of the virus in WSSV-infected *Penaeus monodon* was verified using a immunodot blot assay. Immunodot blot showed a positive reaction in infected shrimp tissues with prominent colour development using 3,3′,5,5′-tetramethylbenzidine (TMB) as a chromogenic substrate when compared with 3–3′ diaminobenzidine tetrahydrochloride (DAB). Highest signal intensities of the immunodots were observed in infected shrimp pleopod extracts and haemolymph. On comparison with polymerase chain reaction (PCR), immunodot blot could detect 76% of PCR-positive WSSV-infected shrimp samples. Immunodot blot was found to be equivalent to first-step PCR sensitivity to detect WSSV particles estimated to contain 1.0×10^5 viral DNA copies.

Keywords: white spot syndrome virus, *Penaeus monodon*, immunodot blot assay, polyclonal

Introduction

Outbreaks of viral disease in *Penaeus monodon* due to the white spot syndrome virus (WSSV) continue to be a major problem for the shrimp aquaculture industry worldwide. Extensive WSSV research is being focused on the development of antiviral therapeutics that can contain this disease. However, the protections conferred by these therapeutics have been found to be shortlived. Presently, with no effective treatments available for the prevention and cure of this disease, good management practice with biosecurity measures is the only option that can reduce the risk of viral outbreaks in shrimps. Major emphasis is being placed on effective monitoring of disease outbreak. Simple and rapid diagnostic tests help in adopting suitable preventive measures against disease occurrence. Several diagnostic techniques such as histopathology (Pantoja & Lightner 2003), polymerase chain reaction (PCR) (Lo, Leu, Ho, Chen, Peng, Chen, Chou, Yeh, Huang, Chou, Wang & Kou 1996; Takahashi, Itami, Maeda, Suzuki, Kasornchandra, Supamattaya, Khongpradit, Boonyaratpalin, Kondo, Kawai, Kusuda, Hirono & Aoki 1996), immunoblot (Poulos, Pantoja, Bradley, Aguilar & Lightner 2001; Anil, Shankar & Mohan 2002; Yoganandhan, Musthaq, Narayanan & Sahul Hameed 2004; Makesh, Koteeswaran, Daniel Joy Chandran, Murali Manohar & Ramasamy 2006), *in situ* hybridization (Kanchanaphum, Wongteerasupaya, Sitidilokratana, Boonsaeng, Panyim, Tassanakajon, Withyachumnarnkul & Flegel 1998) and dot blot (Shekhar, Azad & Ravichandran 2006) have been developed for the detection of WSSV. Among these diagnostic tests, PCR is one of the most sensitive methods that is widely used to detect WSSV in infected shrimp. However, PCR, despite being a very sensitive technique, yields false-positive results if handling of samples is not proper. The contamination risk is high, as many of the nested-PCR methods, using conventional steps, requires transfer of the first step amplified product to a

second PCR reaction tube for the nested amplification (Kiatpathomchai, Boonsaeng, Tassanakajon, Wongteerasupaya, Jitrapakdee & Panyim 2001). In addition, it is also reported that PCR inhibitory factors present in the tissue also have a significant effect on the results (Cunningham 2002; Shekhar *et al.* 2006). In the present study, as an approach to develop a field-based diagnostic test that can avoid the use of sophisticated equipments like a thermocycler, an immunodot blot assay was evaluated.

VP28, which is located on the surface of the virus particle, is reported to play a key role in the systemic WSSV infection in shrimp (van Hulten, Witteveldt, Snippe & Vlak 2001). We therefore chose to clone and express the recombinant protein of WSSV VP28 to raise the polyclonal antibodies against this protein. The polyclonal antisera raised against WSSV VP28 were effective in detecting the presence of the virus in a large number of infected shrimp samples by an immunodot blot with a sensitivity equivalent to first-step PCR.

Materials and methods

Shrimp samples

Ninety-nine white spot-infected *P. monodon* shrimps were collected from shrimp farms located in the east coast regions of India that were affected by disease outbreaks. The shrimp samples were collected in sealed polythene bags and transported in ice to the lab and were stored at − 80 °C till further use.

Preparation of tissue homogenate

WSSV-infected *P. monodon* tissues (approximately 30 mg) were homogenized in 300 μL of extraction buffer (10 mM Tris pH 8.0, 25 mM EDTA, 100 mM NaCl, 0.5% SDS, 100 μg mL^{-1} Proteinase K). The homogenized tissue samples were boiled in a water bath for 2 min, followed by centrifugation at 11 000 *g* for 5 min at room temperature. One microlitre of the supernatant was used for dot blot and PCR analysis. Tissue extract supernatants were also prepared from healthy shrimp samples, which were included as negative controls in the immunodot blot analysis of samples.

PCR

The PCR protocol consisted of an initial denaturation at 95 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 58 °C for 30 s, 72 °C for 30 s and a final extension step of 72 °C for 10 min. The outer and inner primers for the detection of WSSV by nested PCR were based on the sequence reported by Takahashi *et al.* (1996), whereas the primers for amplification of VP28 gene for recombinant protein expression were based on GenBank accession no. DQ681069. The sequences of these primers are shown in Table 1.

Cloning and expression of VP28 gene

To clone the VP28 gene fragment, *Nco*I and *Hind*III restriction sites were added, respectively, to the forward and reverse primers used for amplification of a full-length (615 bp) VP28 gene. The PCR product containing the restriction sites was digested and cloned into the *Nco*I and the *Hind*III sites of the pET32a(+) expression vector (Novagen, Darmstadt, Germany). The plasmid construct was confirmed for the presence of the insert by PCR and restriction digestion with *Nco*I and *Hind*III restriction enzymes. The plasmid was transformed into *Escherichia coli* strain BL21 (DE3) pLysS for protein expression. The transformed cells were grown in Luria–Bertani medium supplemented with ampicillin until the optical density ($OD_{600\,nm}$) reached 0.6. Protein expression was induced for 3–4 h by adding 1.0 mM of isopropyl-β-D-thiogalacto-pyranoside (IPTG). Expression of recombinant protein was analysed on SDS-PAGE, followed by electroelution using an electroeluter (Model 422, Biorad, Hercules, CA, USA).

Table 1 Primers used for white spot syndrome virus (WSSV) screening and cloning of WSSV VP28 gene

	WSSV Primer sequence	Amplified product Size (bp)	Position in nucleotide sequence	References
WSSV (outer)	F: 5′ GACAGAGATATGCACGCCAA 3′ R: 5′ ACCAGTGTTTCGTCATGGAG 3′	643	51–70 674–693	Takahashi *et al.* (1996)
WSSV (inner)	F: 5′ GGTAGATTCTGGTATTAGG 3′ R: 5′ ACCTGGCGTAGTTCTTGC 3′	298	122–140 402–419	Takahashi *et al.* (1996)
VP28	F: 5′ ATGGATCTTTCTTTCACTCT 3′ R: 5′ TTACTCGGTCTCAGTGCCAG 3′	615	1–20 596–615	DQ681069

Production of antiserum

The electroeluted recombinant WSSV VP28 (39.9 kDa) protein was used for immunization in New Zealand white rabbits for raising polyclonal hyper immune serum. The rabbits were first immunized with 300 μg of recombinant protein emulsified with Freund's complete adjuvant, followed by two booster doses of 200 μg each of protein emulsified with Freund's incomplete adjuvant at 30 and 45 days. Blood was collected 20 days after the last booster dose and the serum was separated. The immunoglobulin G (IgG) fraction was purified by protein A affinity chromatography.

Western blotting

Western blots were performed according to Sambrook, Fritsch and Maniatis (1989). The recombinant fusion protein expressed from pET32a(+) expression vector was detected using the Western Breeze chromogenic Western blot immunodetection kit, with alkaline phosphatase-conjugated anti-His mouse monoclonal antibodies (1:10 000 dilution) (Invitrogen, Carlsbad, CA, USA). Western blot was also carried out to check the specificity of the polyclonal antibodies raised against recombinant WSSV VP28 protein using horseradish peroxidase (HRP)-conjugated polyclonal antibodies (1:5000 dilution). Custom conjugation of the primary antibody with HRP was carried out by Bangalore Genei (Bangalore, India). The membranes were developed using BCIP/NBT or TMB as substrates.

Immunodot blot

An immunodot blot assay was performed by spotting 1.0 μL of homogenized tissue sample supernatants of uninfected and infected shrimps made in extraction buffer onto a nitrocellulose membrane. The spots were air dried and were blocked in blocking buffer (50 mM Tris, 200 mM NaCl, 0.05% Tween 20, 3% BSA) for 1 h. The membranes were washed in washing buffer (50 mM Tris, 200 mM NaCl), followed by incubation with anti-WSSV VP28 IgG conjugated with HRP (1:5000 dilution) for 1 h. The membranes were washed thrice for 10 min each with washing buffer before detecting with either TMB or DAB substrate.

Sensitivity of immunodot blot

Detection of purified WSSV recombinant protein at different dilutions was carried out by immunodot blots using HRP-conjugated polyclonal antibodies. The immunodot blots were developed using DAB and TMB as chromogenic substrates for comparative analysis. In the case of infected shrimp samples, the supernatants of different shrimp tissues (muscle, gills, pleopod and eye stalk) and haemolymph at various dilutions were used for the initial analysis of dot-blot sensitivity using HRP-conjugated polyclonal antibodies. The immunodot blot was developed with TMB as a chromogenic substrate. After immunodot blot sensitivity determination of different tissues, the supernatants of shrimp pleopod extracts were chosen for immunodot blot analysis of all the WSSV-infected *P. monodon* field samples. The sensitivity of the immunodot blot was assessed by the quantification of WSSV virus using real-time PCR (Applied Biosystems, Foster City, CA, USA) and nested PCR.

Results

Cloning and expression of the VP28 gene

The positive clones were confirmed for the presence of the insert by PCR and restriction digestion with *Nco*I and *Hind*III restriction enzymes. The PCR amplification of the VP28 WSSV gene resulted in an expected size of 615 bp amplified product. A similar-size insert could be obtained from the vector on *Nco*I and *Hind*III restriction enzyme digestion as shown in Fig. 1(a). On IPTG induction, the expression of the fusion recombinant protein was observed with an expected size of 39.9 kDa, which was purified by electroelution as shown in Fig. 1(b).

Western blotting

The expression of recombinant fusion protein was confirmed by detection of a single band using alkaline phosphatase-conjugated anti-His mouse monoclonal antibodies Fig. 2(a). The specificity of the polyclonal antibodies raised against recombinant and electroeluted WSSV VP28 protein using HRP-conjugated polyclonal antibodies was revealed by the development of a single band as shown in Fig. 2(b).

Sensitivity of immunodot blot

Immunodot blot was carried out using supernatants of the infected shrimp tissue extracts of muscle, gills, pleopod, eye stalk and haemolymph at different dilutions. Pleopod and haemolymph extracts up to 1:100

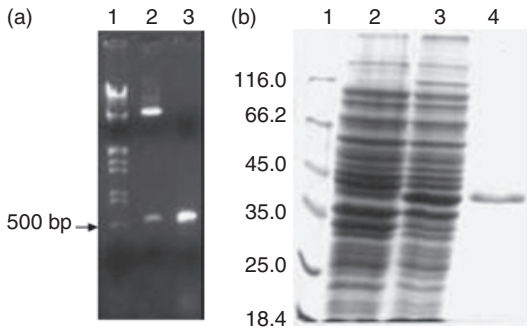

Figure 1 (a) Confirmation of recombinant WSSV VP28-positive clones for the presence of the insert. Lane 1. Lambda DNA *Eco*RI/*Hind*III marker. Lane 2. Restriction digestion of pET32a(+) expression vector with *Nco*I and *Hind*III restriction enzymes. Lane 3. PCR-amplified product of the WSSV VP28 gene (615 bp). (b). Expression of the fusion recombinant protein. Lane 1. Protein molecular weight marker. Lane 2. Uninduced *Escherichia coli* cells (negative control). Lane 3. IPTG-induced *E. coli* cells. Lane 4. Purified recombinant protein (39.9 kDa).

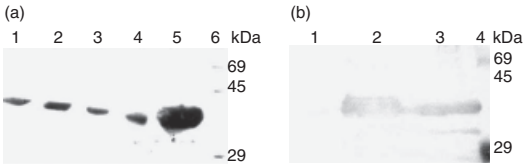

Figure 2 (a) Western blot analysis of expressed WSSV VP28 recombinant protein at different dilutions using alkaline phosphatase-conjugated anti-His mouse monoclonal antibodies. Lane 1. 1:40 protein dilution. Lane 2. 1:30 protein dilution. Lane 3. 1:20 protein dilution. Lane 4. 1:10 protein dilution. Lane 5. Undiluted protein. Lane 6. Coloured protein molecular weight marker. (b). The specificity of the polyclonal antibodies raised against recombinant WSSV VP28 protein using horseradish peroxidase-conjugated polyclonal antibodies. Lane 1. Uninduced *Escherichia coli* cells (negative control). Lane 2. IPTG-induced *E. coli* cells of expressed WSSV VP28 recombinant protein. Lane 3. Purified WSSV VP28 recombinant protein. Lane 4. Coloured protein molecular weight marker.

dilutions showed positive reactions with prominent immunodots using HRP-conjugated polyclonal antibodies developed with a TMB substrate as shown in Fig. 3(a). Supernatants of the WSSV-infected shrimp pleopod extracts were therefore further used for immunodot blot, real-time PCR and nested PCR analysis.

The sensitivity of the immunodot blot was assessed by quantification of the WSSV virus using real-time PCR (Applied Biosystems). A positive reaction of immunodot blot was observed in the shrimp tissue extract estimated to contain 1×10^5 viral DNA copies μL^{-1} as shown in Fig. 3(b). First- and second-step PCR was also carried out in the shrimp-infected tissue extracts at different dilutions using WSSV external and internal primers. First-step PCR was able to detect WSSV (643 bp) in the infected shrimp tissue extract at the dilution estimated to contain 1×10^5 viral DNA copies μL^{-1} or more, whereas nested PCR (298 bp) was positive for WSSV in the infected shrimp tissue extract estimated to contain 1×10^2 viral DNA copies μL^{-1}.

Analysis of WSSV-infected field samples by immunodot blot and PCR

Pleopod tissue extracts from 99 WSSV-infected shrimp samples collected from disease outbreak were used for the detection of virus by immunodot blot. These samples were screened for the presence of WSSV by nested PCR reactions. Seventy-six samples tested first-step PCR positive, showing amplification of a 643 bp product. Twenty samples yielded second-step PCR-positive reactions showing 298 bp amplified PCR products. Three of the shrimp samples tested negative by nested PCR. All these samples were used for subsequent immunodot blot analysis. Initial experiments were conducted on some of the samples in which immunodot blots were developed using DAB or TMB as a substrate. It was observed that the use of TMB led to the development of intense colour and produced high signals for the positive immunodots in comparison with DAB as shown in Fig. 4(a and b). The sensitivity of TMB was found to be high in detecting purified WSSV recombinant protein at a 1:1000 dilution estimated to contain $2\,ng\,\mu L^{-1}$ protein concentration in comparison with DAB, which showed a detection level up to a 1:10 dilution estimated to contain $200\,ng\,\mu L^{-1}$ of viral protein

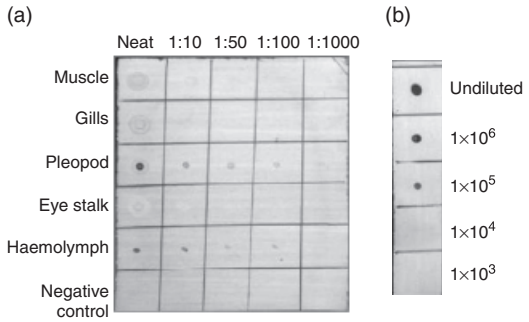

Figure 3 (a) Immunodot blot of WSSV-infected shrimp tissues extracts at different dilutions. (b) The sensitivity of the immunodot blot was assessed by quantification of the WSSV virus in WSSV-infected shrimp tissue extract.

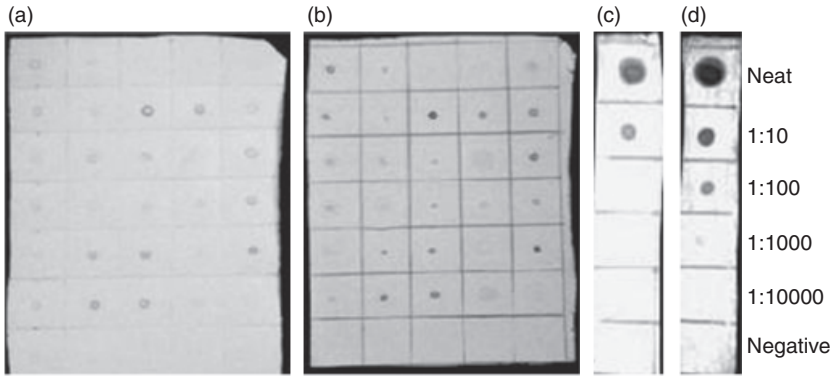

Figure 4 Comparative analysis of TMB and DAB sensitivity in immunodot blot. (a) Screening of samples for the presence of WSSV using DAB as a substrate (b) Screening of samples for the presence of WSSV using TMB as a substrate. (c) Detection of purified WSSV recombinant protein at different dilutions using DAB as a substrate. (d) Detection of purified WSSV recombinant protein at different dilutions using TMB as a substrate.

(Fig. 4c and d). Subsequent analyses of field samples for immunodot blot were continued with the use of TMB as a substrate. Out of 99 total shrimp samples analysed by immunodot blot, 73 of the samples tested positive and 26 samples tested negative for immunodot blot (Fig. 5). The 73 dot-blot-positive samples included 60 and 13 samples that were first- and second-step PCR-positive samples respectively. The 26 samples that tested negative for immunodot blot included 16 samples that were first-step PCR positive, seven samples that were second-step PCR positive and three shrimp samples that tested negative for nested PCR. A total of 76% of the PCR-positive samples could therefore be detected by immunodot blot for WSSV infection.

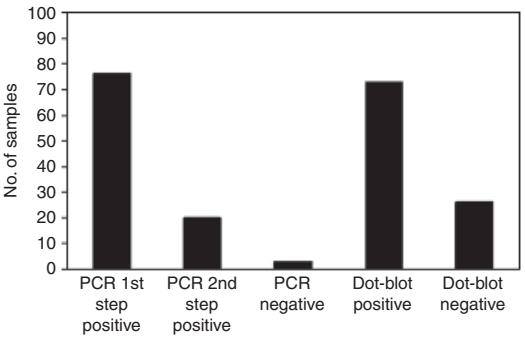

Figure 5 Comparative analysis of PCR and immunodot blot on 99 shrimp samples showing 73 and 26 number of samples testing positive and negative for immunodot blot respectively. Seventy-six and 20 samples testing first-step and second-step PCR positive respectively. Three shrimp samples tested negative by PCR.

Discussion

An early detection of the WSSV virus by a sensitive and accurate molecular diagnosis helps in maintaining a healthy brood stock and in the production of good-quality shrimp larvae. Polymerase chain reaction is one of the most widely used techniques applied to detect WSSV in farmed and wild shrimp, postlarvae, carrier animals, water and pond sediments. As an alternative to PCR, the progress in the use of immuno-based detection methods for shrimp viruses has been rather slow. This is primarily because of the difficulties associated with the availability of a purified antigen for immunization and availability of purified antibodies. For example, Nadala Jr, Tapay, Cao and Loh (1997) reported the cross reaction of the polyclonal antibody raised using WSSV purified from infected shrimp tissue and haemolymph with the shrimp tissue protein. These problems can be circumvented to a large extent by the production of recombinant proteins. Bacterial expression systems have been extensively used in the production of recombinant proteins as they help obtain purified overexpressed recombinant proteins that can be used for raising antibodies. The use of *E. coli* has been reported to be effective in producing recombinant proteins of WSSV (Sathish, Musthaq, Sahul Hameed & Narayanan 2004; Yoganandhan *et al.* 2004; Chaivisuthangkura, Phattanapaijitkul, Thammapalerd, Rukpratanporn, Longyant, Sithigorngul & Sithigorngul 2006), with the production of purified recombinant protein up to 10 mg L^{-1} of bacterial culture (You, Nadala, Yang, van Hulten & Loh 2002). Previous research on WSSV has shown that its

envelope protein such as VP28 plays a vital role in the initial steps of the systemic infection of WSSV in shrimp (van Hulten *et al.* 2001). The recombinant VP28 protein expressed in *E. coli* has been successfully utilized with the purpose of vaccinating shrimp (Witteveldt, Cifuentes, Vlak & van Hulten 2004; Witteveldt, Vlak & van Hulten 2004) and for developing monoclonal (Liu, Wang, Tian, Yin & Kwang 2002) and polyclonal antibodies (You *et al.* 2002; Yoganandhan *et al.* 2004) for use in immuno-based detection tests. In the present study, 1.0 mg of the overexpressed purified recombinant protein of WSSV VP28 protein (39.9 kDa) in *E. coli* cells was sufficient for raising polyclonal antibodies in New Zealand white rabbit. The identity of the recombinant protein was confirmed with anti-His antibody by Western blotting.

Several reports have shown that PCR is the most sensitive technique when compared with different molecular techniques that have been used for detection of WSSV such as DNA dot blot (Shekhar *et al.* 2006) and WSSV test strip (Sithigorngul, Rukpratanporn, Pecharaburanin, Longyant, Chaivisuthangkura & Sithigorngul 2006).

Shrimple, an immunochromatographic detection assay for WSSV, is reported to be less sensitive in detecting the early onset of infection when compared with real-time PCR. Only 34.7% of the specimens tested were determined to be positive for WSSV using the shrimple test; however, the assay was found to be sensitive enough to detect WSSV infections before development of gross signs of acute disease (Powell, Burge, Browdy & Shepard 2006). In the present study, the sensitivity to detect WSSV with the use of first-step PCR (1×10^5 viral DNA copies μL^{-1}) and nested PCR (1×10^2 viral DNA copies μL^{-1}) is in agreement with other reports showing nested PCR to be more sensitive than first-step PCR by approximately 10^3–10^4-fold (Sritunyalucksana, Srisala, McColl, Nielsen & Flegel 2006; Srisala, Tacon, Flegel & Sritunyalucksana 2008). Shrimp tissue extract dilutions $< 1 \times 10^5$ viral DNA copies μL^{-1} resulted in second-step PCR-positive reactions for WSSV. At these dilutions, the nested PCR was found to be more sensitive in comparison with immunodot blot, which failed to detect WSSV. Even though immunodot blot was found to be less sensitive as compared with the nested PCR, its sensitivity was found to be equivalent to first-step PCR in WSSV detection in tissue extracts having 1×10^5 viral DNA copies μL^{-1}. Similar observations of monoclonal-based immunodetection tests with a diagnostic sensitivity comparable to single-step PCR for the detection of WSSV have been reported (Anil *et al.* 2002; Makesh *et al.* 2006). The monoclonal antibodies specific to VP28 of WSSV could detect 500 pg protein by immunoperoxidase dot blotting (Anil *et al.* 2002) and 625 pg by the immunocomb detection method (Makesh *et al.* 2006).

Among the HRP chromogens, DAB and TMB are widely used for histochemistry. However, several studies have reported that DAB is less sensitive than the TMB (Mesulam 1978; Morrell, Greenberger & Pfaff 1981; van der Want, Klooster, Nunes–Cardozo, de Weerd & Liem 1997). In the case of shrimp viruses, the use of these two substrates for HRP has been evaluated for sensitivity to detect virus by dot immunoassay. TMB, which could detect 0.4 ng of shrimp yellow head viral protein, was found to be more sensitive when compared with DAB, having a detection limit of 0.8 ng of viral protein (Lu, Tapay & Loh 1996). In the present study, similar results of enhanced sensitivity of TMB (2 ng μL^{-1}) with intense colour development in detecting purified WSSV recombinant protein were observed when compared with DAB (200 ng μL^{-1}). The sensitivity of immunodot blot assay in the present study was observed to be in the same range as reported by others (800–5 ng of the total protein concentration) for the detection of the virus in WSSV-infected shrimp samples (Sahul Hameed, AnilKumar, Stephen Raj & Jayaraman 1998; You *et al.* 2002; Yoganandhan *et al* 2004).

One of the major advantages of this immunodot blot detection method is its ability to detect and grade the severity of viral infections. Grading of infections is one of the important criteria required for the proper management of WSSV in culture systems (Lo, Chang, Cheng & Kou 1998). The use of single-tube semi nested PCR has been reported for grading the severity of WSSV infections in *P. monodon*. For example, heavy ($> 2 \times 10^4$ viral particles), moderate (around 2×10^3 viral particles) and light infections (20–200 viral particles) of WSSV have been graded based on multiplex PCR (Kiatpathomchai *et al.* 2001). In our earlier studies using single tube nested PCR, we have also reported the level of WSSV infection in different tissues of *P. monodon*. Cuticle and telson of shrimp were reported to be the most severely infected tissues, followed by moderately infected gill tissue, and mild infection was found in other tissues such as the eye stalk, pleopod, periopods and uropod (Shekhar *et al.* 2006). Similarly, immunodot blot assay has been used for the detection of virus in various WSSV-infected shrimp organs such as eyestalks, head muscle, gills, heart, haemolymph, tail and appendages. The haemolymph was found to be a good source for the

early detection of WSSV at 12 h post infection (Yoganandhan *et al* 2004) by immunodot blot assay. The tail, head tissue and eye stalk of WSSV-infected shrimp were observed to contain a high viral load by Western blot and ELISA (Sathish, Selvakumar, Sahul Hameed & Narayanan 2004). Gill homogenate of WSSV-infected shrimp is also reported to be effective in WSSV detection by immunodot blot assay (You *et al.* 2002). In this study, we used WSSV-infected muscle, gills, pleopod, eye stalk and haemolymph for the detection of WSSV by immunodot blot assay. Both haemolymph and pleopods were found to be suitable for the detection of virus at a 1:100 dilution in generating a positive reaction with a distinct colour development when compared with other tissue homogenates.

Because the technique in the present study has the ability to detect WSSV in samples collected from disease outbreak having a viral load with a sensitivity equivalent to first-step PCR (1×10^5 viral DNA copies μL^{-1}), this may be used for the initial screening of a large number of samples avoiding the use of first-step PCR. Furthermore, it has been reported that single-step PCR is a good indicator of field outbreaks than nested PCR (Lo *et al.* 1998; Tsai, Kou, Liu, Liu, Chang, Peng, Hsu, Wang & Lo 1999). Good management practice suggests monitoring for WSSV by a single-step and nested PCR at regular intervals during the shrimp cultivation period. The use of nested PCR as a technique to screen and grade WSSV infections into categories ranging from very severe, severe, light and very light infections helps in providing an ample opportunity to decide on any emergency harvest if needed. Usually, an emergency harvest is suggested if the shrimps are diagnosed positive by single-step PCR.

About 76.04% of the nested PCR-positive samples could be detected by immunodot blot in the present study. We have successfully circumvented the need to use first-step PCR using a simplified immunodot blot assay. The tissue extraction procedure described here requires minimal time, efforts and yields satisfactory results under the given conditions of our assay. Our method with visual estimation of results in shrimps would be more suitable and applicable for routine assays before full-blown WSSV outbreaks without the use of sophisticated equipments, which would help in adopting suitable preventive measures at the field level.

References

Anil T.M., Shankar K.M. & Mohan C.V. (2002) Monoclonal antibodies developed for sensitive detection and comparison of white spot syndrome virus isolates in India. *Diseases of Aquatic Organisms* **51**, 67–75.

Chaivisuthangkura P., Phattanapaijitkul P., Thammapalerd N., Rukpratanporn S., Longyant S., Sithigorngul W. & Sithigorngul P. (2006) Production of polyclonal antibodies against recombinant VP26 structural protein of white spot syndrome virus (WSSV). *Science Asia* **32**, 201–204.

Cunningham C.O. (2002) Molecular diagnosis of fish and shellfish diseases: present status and potential use in disease control. *Aquaculture* **206**, 19–55.

Kanchanaphum P., Wongteerasupaya C., Sitidilokratana N., Boonsaeng V., Panyim S., Tassanakajon A., Withyachumnarnkul B. & Flegel T.W. (1998) Experimental transmission of white spot syndrome virus (WSSV) from crabs to shrimp *Penaeus monodon*. *Disease of Aquatic Organisms* **34**, 1–7.

Kiatpathomchai W., Boonsaeng V., Tassanakajon A., Wongteerasupaya C., Jitrapakdee S. & Panyim S. (2001) A non-stop, single-tube, semi-nested PCR technique for grading the severity of white spot syndrome virus infections in *Penaeus monodon*. *Disease of Aquatic Organisms* **47**, 235–239.

Liu W., Wang Y.T., Tian D.S., Yin Z.C. & Kwang J. (2002) Detection of white spot syndrome virus (WSSV) of shrimp by means of monoclonal antibodies (MAbs) specific to an envelope protein (28 kDa). *Disease of Aquatic Organisms* **49**, 11–18.

Lo C.F., Chang Y.S., Cheng C.T. & Kou G.H. (1998) Monitoring cultured shrimp for white spot syndrome virus (WSSV) infection during their growth period by polymerase chain reaction. In: *Advances in Shrimp Biotechnology* (ed. By T.W. Flegel), pp. 281–286. National Center for Genetic Engineering and Biotechnology, Bangkok, Thailand.

Lo C.F., Leu J.H., Ho C.H., Chen C.H., Peng S.E., Chen Y.T., Chou C.M., Yeh P.Y., Huang C.J., Chou H.Y., Wang C.H. & Kou G.H. (1996) Detection of baculovirus associated with white spot syndrome (WSBV) in *penaeid* shrimps using polymerase chain reaction. *Disease of Aquatic Organisms* **25**, 133–141.

Lu Y., Tapay L.M. & Loh P.C. (1996) Development of a nitrocellulose-enzyme immunoassay for the detection of yellow-head virus from penaeid shrimp. *Journal of Fish Diseases* **19**, 9–13.

Makesh M., Koteeswaran A., Daniel Joy Chandran N., Murali Manohar B. & Ramasamy V. (2006) Development of monoclonal antibodies against VP28 of WSSV and its application to detect WSSV using immunocomb. *Aquaculture* **261**, 64–71.

Mesulam M.M. (1978) Tetramethylbenzidine for horseradish peroxidase neurohistochemistry: a noncarcinogenic blue reaction product with superior sensitivity for visualizing neural afferents and efferents. *Journal of Histochemistry and Cytochemistry* **26**, 106–117.

Morrell J.I., Greenberger L.M. & Pfaff D.W (1981) Comparison of horseradish peroxidase visualization methods: quanti-

tative results and further technical specifics. *Journal of Histochemistry and Cytochemistry* **29**, 903–916.

Nadala E.C.B. Jr, Tapay L.M., Cao S. & Loh P.C. (1997) Detection of Yellow head virus and Chinese baculovirus in *penaeid* shrimp by western blot technique. *Journal of Virological Methods* **69**, 39–44.

Pantoja C.R. & Lightner D.V. (2003) Similarity between the histopathology of white spot syndrome virus and yellow head syndrome virus and its relevance to diagnosis of YHV disease in the Americas. *Aquaculture* **218**, 47–54.

Poulos B.T., Pantoja C.R., Bradley D.D., Aguilar J. & Lightner D.V. (2001) Development and application of monoclonal antibodies for the detection of white spot syndrome virus of penaeid shrimp. *Diseases of Aquatic Organisms* **47**, 13–23.

Powell J.W.B., Burge E.J., Browdy C.L. & Shepard E.F. (2006) Efficiency and sensitivity determination of Shrimple®, an immunochromatographic assay for white spot syndrome virus (WSSV), using quantitative real-time PCR. *Aquaculture* **257**, 167–172.

Sahul Hameed A.S., AnilKumar M., Stephen Raj M.L. & Jayaraman K. (1998) Studies on the pathogenicity of systemic ectodermal and mesodermal baculovirus and its detection in shrimp by immunological methods. *Aquaculture* **160**, 31–45.

Sambrook J., Fritsch E.F. & Maniatis T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sathish S., Musthaq S., Sahul Hameed A.S. & Narayanan R.B. (2004) Production of recombinant structural proteins from the Indian WSSV isolate. *Aquaculture* **242**, 69–80.

Sathish S., Selvakumar C., Sahul Hameed A.S. & Narayanan R.B. (2004) 18-kDa protein as a marker to detect WSSV infection in shrimps. *Aquaculture* **238**, 39–50.

Shekhar M.S., Azad I.S. & Ravichandran P. (2006) Comparison of dot blot and PCR diagnostic techniques for detection of white spot syndrome virus in different tissues of *Penaeus monodon*. *Aquaculture* **261**, 1122–1127.

Sithigorngul W., Rukpratanporn S., Pecharaburanin N., Longyant S., Chaivisuthangkura P. & Sithigorngul P. (2006) A simple and rapid immunochromatographic test strip for detection of white spot syndrome virus (WSSV) of shrimp. *Disease of Aquatic Organisms* **72**, 101–106.

Srisala J., Tacon P., Flegel T.W. & Sritunyalucksana K. (2008) Comparison of white spot syndrome virus PCR-detection methods that use electrophoresis or antibody-mediated lateral flow chromatographic strips to visualize PCR amplicons. *Journal of Virological Methods* **153**, 129–133.

Sritunyalucksana K., Srisala J., McColl K., Nielsen L. & Flegel T.W. (2006) Comparison of PCR testing methods for white spot syndrome virus (WSSV) infections in penaeid shrimp. *Aquaculture* **255**, 95–104.

Takahashi Y., Itami T., Maeda M., Suzuki N., Kasornchandra J., Supamattaya K., Khongpradit R., Boonyaratpalin S., Kondo M., Kawai K., Kusuda R., Hirono I. & Aoki T. (1996) Polymerase chain reaction (PCR) amplification of bacilliform virus (RV-PS) DNA in *Penaeus japonicus* Bate and Systemic ectodermal and mesodermal baculovirus (SEMBV) DNA in *Penaeus monodon* Fabricius. *Journal of Fish Disease* **19**, 399–403.

Tsai M.F., Kou G.H., Liu H.C., Liu K.F., Chang C.F., Peng S.E., Hsu H.C., Wang C.H. & Lo C.F. (1999) Long-term presence of white spot syndrome virus (WSSV) in a cultivated shrimp population without disease outbreaks. *Disease of Aquatic Organisms* **38**, 107–114.

van der Want J.J.L., Klooster J., Nunes–Cardozo B., de Weerd H. & Liem R.S.B. (1997) Tract-tracing in the nervous system of vertebrates using horseradish peroxidase and its conjugates: tracers, chromogens, and stabilization for light and electron microscopy. *Brain Research Protocols* **1**, 267–279.

van Hulten M.C., Witteveldt J., Snippe M. & Vlak J.M. (2001) White spot syndrome virus envelope protein VP28 is involved in the systemic infection of shrimp. *Virology* **285**, 228–233.

Witteveldt J., Cifuentes C.C., Vlak J.M. & van Hulten M.C.W. (2004) Protection of *Penaeus monodon* against white spot syndrome virus by oral vaccination. *Journal of Virology* **78**, 2057–2061.

Witteveldt J., Vlak J.M. & van Hulten M.C.W. (2004) Protection of *Penaeus monodon* against white spot syndrome virus using a WSSV subunit vaccine. *Fish and Shellfish Immunology* **16**, 571–579.

Yoganandhan K., Musthaq S.S., Narayanan R.B. & Sahul Hameed A.S. (2004) Production of polyclonal antiserum against recombinant VP28 protein and its application for the detection of white spot syndrome virus in crustaceans. *Journal of Fish Diseases* **27**, 517–522.

You Z.E., Nadala C.B., Yang J., van Hulten M.C. & Loh P.C. (2002) Production of polyclonal antiserum specific to the 27.5 kDa envelope protein of white spot syndrome virus. *Disease of Aquatic Organisms* **51**, 77–80.