Fig. 1

```
               T  I
               S  T  Y  N  F  D  I  T  P  D  N  I  C  P  E  G  V  Y  D  F  E  T
GAV    73-138 UCAACAUACAAUUUCGACAUCACUCCAGACAACAUCUGCCCAGAAGGCGUCUAUGAUUUCGAGACC
YHV           A---U------C--------------------------U-----------------C--------A

                                      GY1                GY2
                              N           V  T
               F  D  S  Y  L  S  F  M  L  T  K  E  R  H  N  I  N  I  D  I  G  T
GAV   343-408 UUCGACUCCUAUCUCUCAUUUAUGCUCACAAAGGAACGCCAUAACAUCAACAUCGACAUUGGUACA
YHV           ---------------AAC--C-----UGUC-CA--G--U--C-----------U-----C--C---

                                     G3 / Y3
                        N           A
               E  R  Y  E  E  V  M  I  G  V  T  R  L  Y  Y  A  Y  Q  Y  D  R  G
GAV   466-531 GAACGUUACGAAGAAGUCAUGAUAGGUGUCACUCGUCUCUACUACGCCUAUCAGUAUGAUCGCGGU
YHV           -----A---A-C---------GCC--G--U-----CU-A--U--U-----C---------A-A---

                                             G6

               K  L  H  E  D  R  H  L  I  R  D  R  L  T  H  M  D  F  T  S  G  Q
GAV   826-891 AAACUACACGAAGACCGUCACCUCAUUCGUGAUCGUCUCACACACAUGGACUUCACAUCAGGACAG
YHV           --------------U--------A--C-----C-----------------------C-----U--A

                                                        GY4
                                          M                                P
               F  K  N  Y  D  R  N  T  G  I  S  C  S  L  T  E  F  Q  L  S  L  S
GAV 1078-1143 UUCAAGAACUAUGACCGAAACACCGGCAUAUCCUGUUCUCUCACUGAAUUCCAGCUCUCUCUCUCG
YHV           -----A-----C---A-------------G-------------------------------C---U

                                                        GY5
```

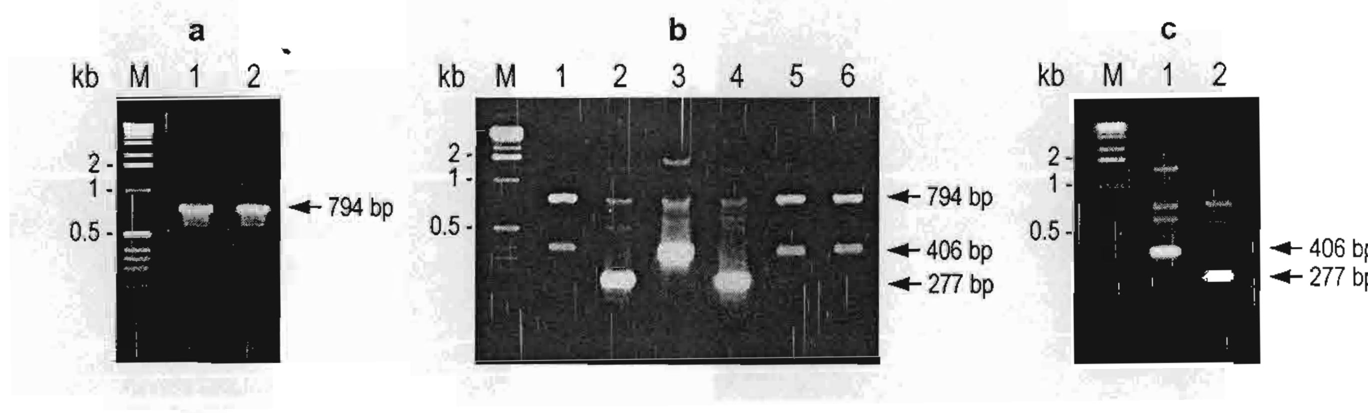
Fig. 6

```
                  G3/Y3                              G6

           S   F   M   L   T   K   E   R       I   G   V   T   R   L   Y   Y
GAV#6     UCA UUU AUG CUC ACA AAG GAA CGC-105-AUA GGU GUC ACU CGU CUC UAC UAC
GAV#7     ... ..C ... ... ... ... ... ...     ... ... ... ..C ..C ... ... ...
GAV#8     ... ..C ... ... ... ... ... ...     ... ... ... ... ..C ... ... ...
GAV#9     ... ..C ... ... ... ... ... ...     .C. ... ... ..C ..C ... ... ...
GAV#10    ... ..C ... ... ... ... ... ...     ... ... ... ..C ..C ... ... ...
GAV#1     ... ..C ... ... ... ... ... ...     ... ... ... ..C ..C ... ... ...
GAV#2     ... ..C ... ... ... ... ... ...     ... ... ... ..C ..C ... ... ...
GAV#3     ... ..C ... ... ... ... ... ...     ... ... ... ... ..C ... ... ...
GAV#4     ... ..C ... ... ... ... ... ...     ... ... ... ..C ..C ... ... ...
GAV#5     ... ..C ... ... ... ... ... ...     ... ... ... ... ..C ... ... ...

           N               V   T               A
YHV#1     AAC ..C ... ..U GUC .CA ..G ..U-105-GCC ..G ..U ... ..C U.A ..U ..U
YHV#2     AAC ..C ... ..U GUC .CA ..G ..U     GCC ..G ..U ... ..C U.A ..U ..U
YHV#3     AAC ..C ... ..U GUY .CA ..G ..U     GCC ..G ..U ... ..C U.A ..U ..U
YHV#4     AAC ..C ... ..U GUC .CA ..G ..U     GCC ..G ..U ..Y ..C U.A ..U ..U
YHV#5     AAC ..C ... ..U GUC .CA ..G ..U     GCC ..G ..U ..Y ..C U.A ..U ..U
YHV#6     AAC ..C ... ..U GUC .CA ..G ..U     GCC ..G ..U ... ..C U.A ..U ..U
YHV#7     AAC ..C ... ..U GUY .CA ..G ..U     GCC ..G ..U ... ..C U.A ..U ..U
YHV#8     AAC ..C ... ..U GUC .CA ..G ..U     GCC ..G ..U ... ..C U.A ..U ..U
YHV#9     AAC ..C ... ..U GUC .CA ..G ..U     GCC ..G ..U ... ..C U.A ..U ..U
```

Figure 2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kb M 1 2
2 -
1 -
0.5 -
794 bp
b
kb M 1 2 3 4 5 6
2 -
1 -
0.5 -
794 bp
406 bp
277 bp
c
kb M 1 2
2 -
1 -
0.5 -
406 bp
277 bp

Figure 3

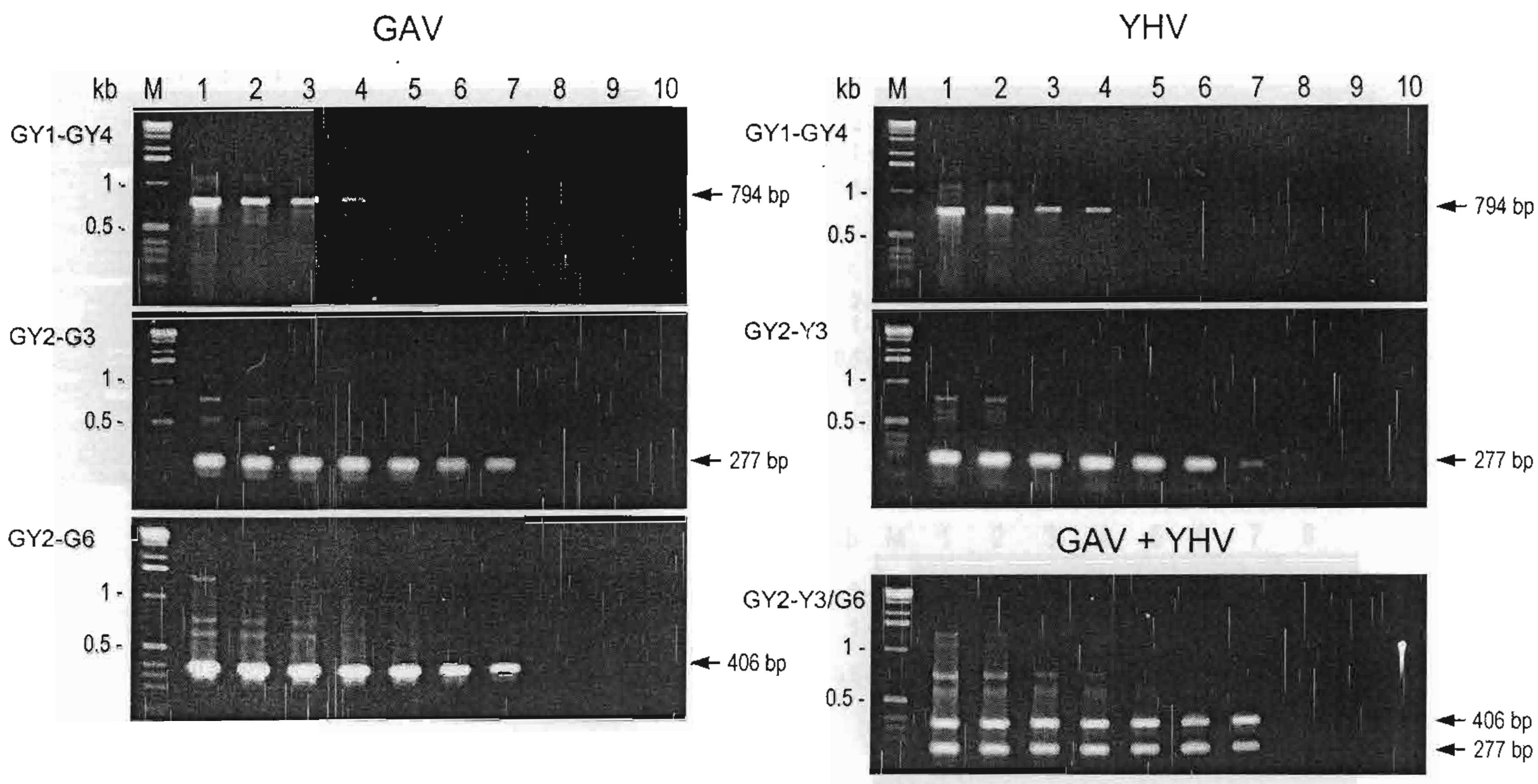

GAV
kb M 1 2 3 4 5 6 7 8 9 10
GY1-GY4
1 -
0.5 -
794 bp
GY2-G3
1 -
0.5 -
277 bp
GY2-G6
1 -
0.5 -
406 bp
YHV
kb M 1 2 3 4 5 6 7 8 9 10
GY1-GY4
1 -
0.5 -
794 bp
GY2-Y3
1 -
0.5 -
277 bp
GAV + YHV
GY2-Y3/G6
1 -
0.5 -
406 bp
277 bp

Figure 4

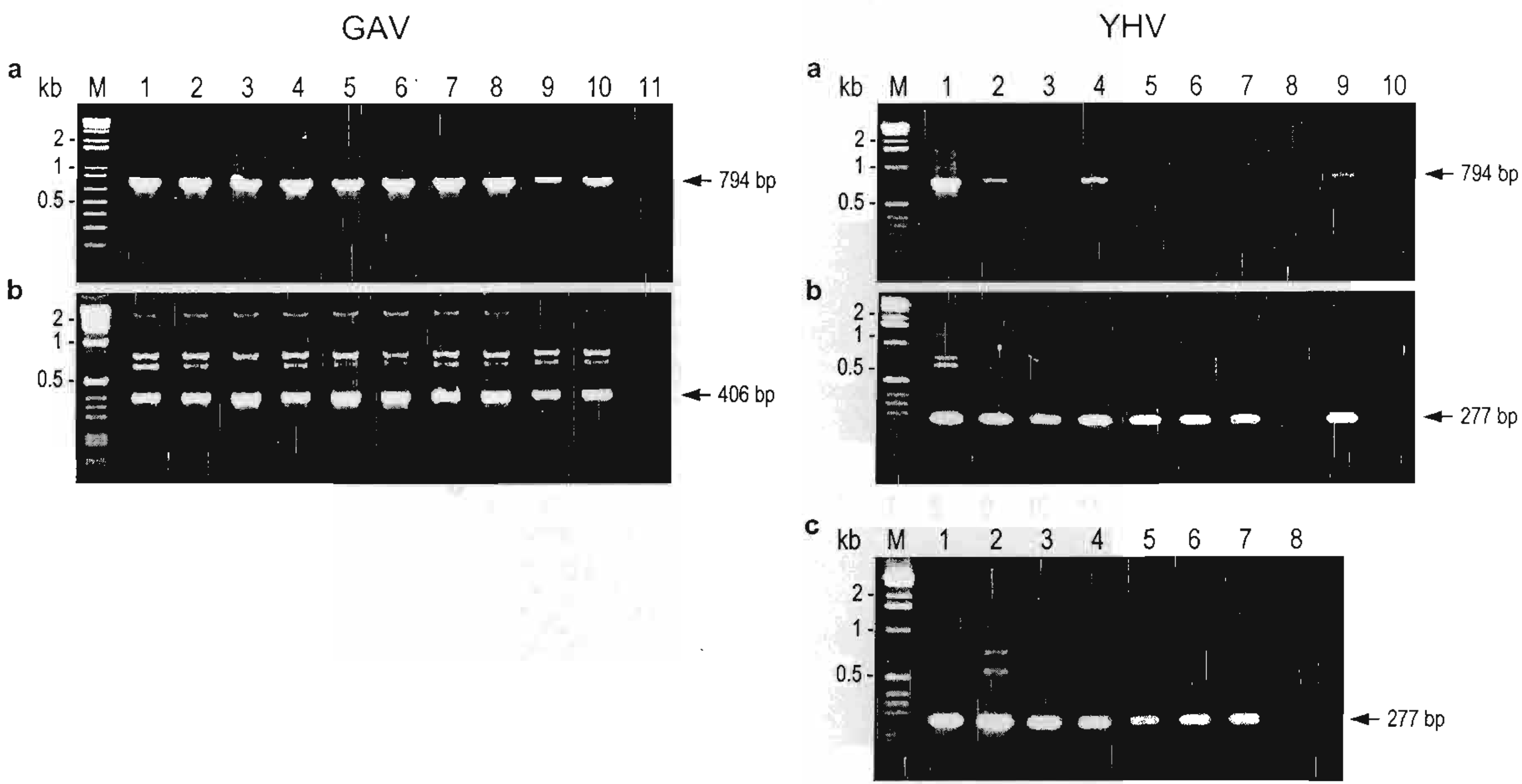
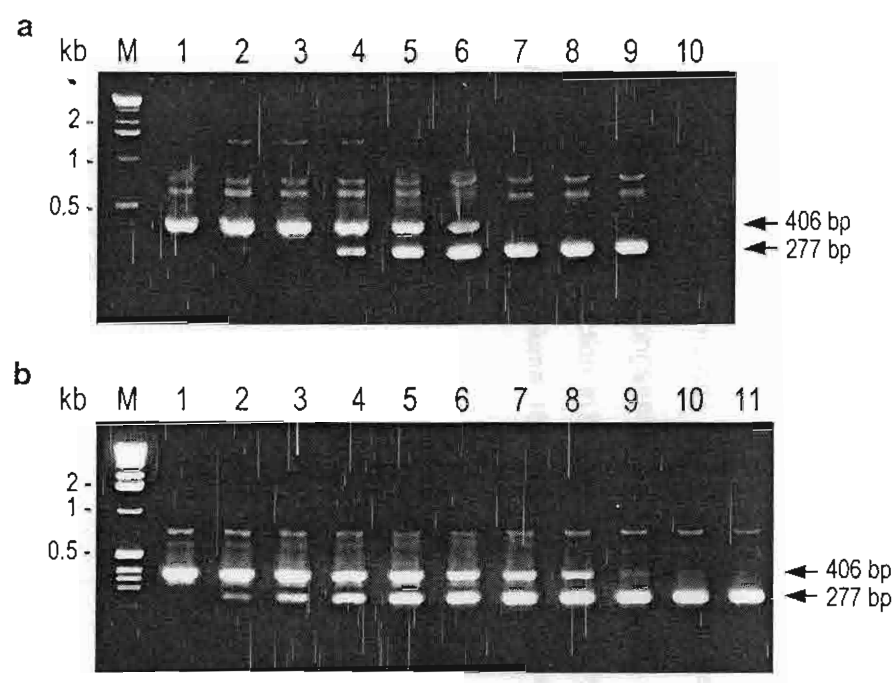


Figure 5



a
kb M 1 2 3 4 5 6 7 8 9 10
2
1
0.5
406 bp
277 bp
b
kb M 1 2 3 4 5 6 7 8 9 10 11
2
1
0.5
406 bp
277 bp

5.3.2

Sukumsirichart W., Kiatpathomchai W., **Wongteerasupaya** C., Withyachumnarnkul B., Flegel TW., Boongsaeng V., and Panyim S. Detection of hapatopancreatic parvoviras (HPV) infection in Penaeus monodon using PCR – ELISA. *Molecular and Cellular Probes* volume 16, August 2002, Pages 409 – 413

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Detection of hepatopancreatic parvovirus (HPV) infection in *Penaeus monodon* using PCR-ELISA

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A rapid and sensitive PCR-ELISA has been developed for detection of hepatopancreatic parvovirus (HPV) in *Penaeus monodon*. The specific primer set amplified 156 bp fragment and could detect as a little as 0·01 fg of purified HPV DNA which equivalent to three viral particles. No cross-reactivity was observed when nucleic acid templates from white spot syndrome virus, yellow-head virus, monodon baculovirus and shrimp were tested. The crude DNA simple prepared from hepatopancreas can be used as DNA template and provide a favorable result. Using this technique for detection of HPV infection in 87 carrier shrimps revealed the higher sensitivity and efficiency of detection when compared to histological examination and conventional PCR. Sixty-two percent infection was detected by PCR-ELISA from samples with HPV negative diagnosed by histological examination. Therefore, this sensitive and specific method is promisingly useful for early detection of HPV infection in broodstock, carriers and for *ex situ* application where large numbers of samples can be analyzed simultaneously.

KEYWORDS: hepatopancreatic parvovirus, PCR-ELISA, *Penaeus monodon*, polymerase chain reaction.

INTRODUCTION

Hepatopancreatic parvovirus (HPV) causing disease in several species of penaeid shrimps including *Penaeus monodon*.[1] The heavy infected shrimp demonstrated of stunting which affected shrimp production of farmers.[2]

A number of diagnostic methods were developed for detection of this virus including histological examination (H&E staining),[3] Transmission electron microscopy (TEM),[1] *in situ* hybridization,[4] and polymerase chain reaction (PCR).[5,6] The classical H&E staining and TEM are reliable techniques but require an experienced and skilful technician. *In situ* hybridization is specific but low sensitivity and the commercial available probe was strain-specific.[4] Polymerase chain reaction (PCR) was highly sensitive but the analysis by agarose gel electrophoresis make it is not suitable for field application which several samples need simultaneously examine.

The PCR-ELISA assay offers an alternative method for detection of PCR products with highly accurate results. The protocol does not require hazardous substances such as ethidium bromide. It has been reported as a sensitive and specific method for detection of bacterial and viral DNA or RNA.[7–1?] This methods with a microtiter plate has been used for the detection of *Mycobacterium tuberculosis*,[?]

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human cytomegalovirus,[8] Dengue virus,[9] human papillomavirus,[10] herpesvirus 6,[11] Plum pox polyvirus (PPV),[12] Rabies viruses,[13] *Coxiella burnetii*,[14] bacteria in cow's milk,[15] *Escherichia coli*,[16] and parvovirus B19.[17]

The technique involves the combination of amplification of viral DNA by PCR using specific primers, hybridization with a specific probe and finally the detection of the hybridized product by the ELISA technique. Besides the safety of non-radioisotopic detection, the method of microtiter plate hybridization has advantages of speed, suitability for a large number of samples and applicability to automation.[10,17] Therefore this studies, we demonstrated that the PCR-ELISA assay is sensitive and specific to use as a diagnostic method for detection HPV infection in *P. monodon*.

MATERIALS AND METHODS

Shrimp samples

Eighty-seven shrimp samples were collected from ponds in the central and southern parts of Thailand including Samut Prakarn, Ratchaburi, Pattanee, and Phuket. The hepatopancreases were removed and transferred immediately to liquid nitrogen and then stored at -80°C. For post-larvae, the whole fry were fixed in 70% ethanol.

HPV DNA preparation

The viral particle was purified from hepatopancreases and DNA was extracted (Sukhumsirichart *et al.*, 1999).[5] For crude DNA preparation, the hepatopancreases or whole post larvae were homogenized in lysis solution containing 0·5 N NaOH–0·025% SDS, boiled for 10 min and then centrifuge at 10,000 rpm for 2 min. The crude lysate (1–5 µl) was then used in PCR amplification.

PCR amplification and DIG-labelling

The HPV DNA was specific amplified and labeled by Digoxygenin-dUTP. The PCR reaction was carried out in 50 µl reaction mixture containing, 5 µl crude DNA template, 1 × PCR reaction buffer (10 mM Tris-HCl-pH 8·0, 50 mM KCl and 1% w/v gelatin), 1·5 mM $MgCl_2$, 200 µM dNTP, 10 µM Digoxigenin-dUTP (DIG) (Boehringer Mannheim), 0·1 µM each of primer 121F (5'...GCA CTT ATC ACT GTC TCT AC...3') and 276R (5'...GTG AAC TTT GTA AAT ACC TTG...3'), 2 unit *Taq* DNA polymerase (Perkin-Elmer). The reaction mixtures were pre-heat at 94°C for 3 min and then performing PCR amplification for 40 cycles as the following condition; denature at 94°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 30 s. The final extension was carried out at 72°C for 5 min. PCR negative controls containing no template DNA were performed for each PCR reaction.

Detection of DIG-labeled HPV DNA using ELISA assay

The DIG-labeled DNA was further detected using ELISA detection kit (Boehringer Mannheim). The labeled PCR product (5 µl) was denatured in 40 µl denaturing solution at room temperature for 10 min. Five hundred microliter of hybridization solution containing 7·5 pmol/ml biotin-labeled specific internal sequence probe (5'...Biotin-AATCCTCCT-CCTTCA TGGTTA...3') was added and well mixed. Aliquots of 200 µl of the mixtures were pipetted into avidin-coated microtite plate and incubated at 37°C for 2 h. At the end of the incubation period, the solution of a given well was discard and washed for 3 times with 250 µl of washing solution. After the last washing step, 200 µl of the anti-digoxigenin peroxidase (Anti-DIG-POD) solution was added and further incubated for 30 min in a 37°C shaker. The solution was then discarded and washed for three times. The microtiter plate was air-dried on a piece of paper. The color was developed by adding 200 µl of ATBS® (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) substrate solution and incubated in the dark for 30 min. The optical density of color product was measured at 405 nm using ELISA photometric measurement apparatus. The intrinsic extinction value of ABTS was evaluated by measuring this solution alone (reagent blank).

Sensitivity and specificity of detection

To determine the sensitivity of PCR-ELISA for detection of HPV DNA, ten fold dilution (1 pg–0·01 fg) of purified HPV DNA was prepared and employed as the DNA templates for PCR-ELISA assay.

Purified or crude DNA prepared from shrimp tissue and other viral pathogens that infect *P. monodon* were tested by PCR-ELISA assay including yellow-head virus (YHV), white-spot syndrome virus (WSSV) and monodon baculovirus (MBV)-infected hepatopancreases.

Southern hybridization

Ten-fold serially dilution of purified HPV DNA ranging from 100 to 001 fg was subjected to PCR amplification for 40 cycles as described above. The PCR products (15 μl) were analyzed by agarose gel electrophoresis, transferred onto nylon membrane and hybridized with DIG-labeled 156 bp DNA fragment as a probe.[18]

RESULTS

Sensitivity and specificity of PCR-ELISA for detection of HPV

The sensitivity of PCR-ELISA for the detection of HPV DNA was determined in comparison to agarose gel electrophoretic analysis and Southern blot hybridization. A ten-fold serial dilution of HPV DNA was prepared and subjected to PCR amplification. The DIG-labeled 156 bp DNA fragment was then analyzed by agarose gel electrophoresis and ELISA. Results in Figures 1A and B showed that the sensitivity of detection by agarose gel electrophoresis was 1 fg, whereas 100 times higher sensitivity (0·01 fg) was obtained by ELISA. This sensitivity was 10 times higher than Southern blot hybridization (C).

The amount of HPV DNA in samples were also determined by measuring optical density of the color product at 405 nm (OD_{405}). The result obtained in Figure 2 demonstrates the ability of detecting as a little as 0·01 fg of purified HPV DNA which equivalent to three viral particles and agree with the sensitivity of detection in Figure 1B when the color was detected by naked eyes.

The specificity of the primer and internal sequence probe used in the PCR-ELISA reaction were tested using nucleic acid of white syndrome virus (WSSV), shrimp, yellow-head virus (YEV), monodon baculovirus (MBV) and shrimp. The result in Figure 2 revealed that the primers and probe were specific only for HPV infection, the optical density at 405 nm of other viruses and shrimp were less than the mean absorbance of negative control (0·026 ± 3SD).

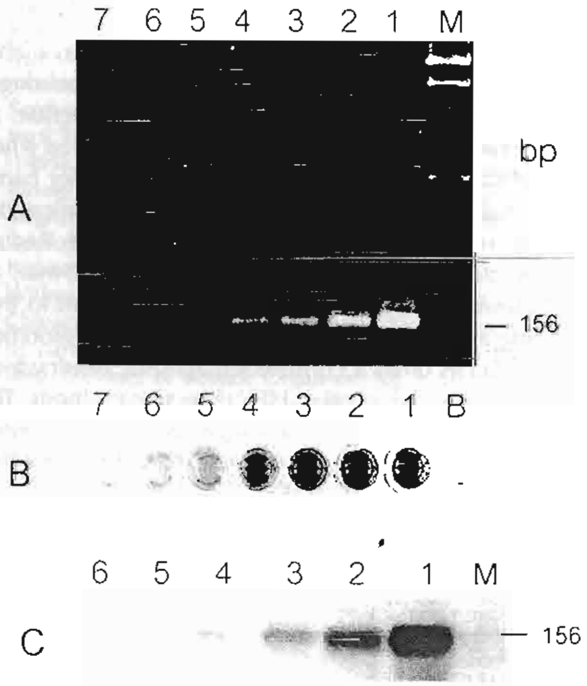

Fig. 1. Comparison of sensitivity for detection of HPV DNA by agarose gel electrophoresis, PCR-ELISA and southern blot hybridization. Ten-fold serial dilution of HPV DNA ranging from 1 pg to 0·01 fg (lanes 1–6) was individually subjected to PCR amplification. The 156 bp PCR product was analyzed by agarose gel electrophoresis (A) and ELISA assay (B). Southern blot hybridization of serial dilution of HPV DNA ranging from 100 to 0·01 fg (lanes 1–6) using DIG-labeled 156 bp fragment as probe was determined (C). Lane M represent DNA marker. Lanes 7 and B were negative control and reagent blank, respectively.

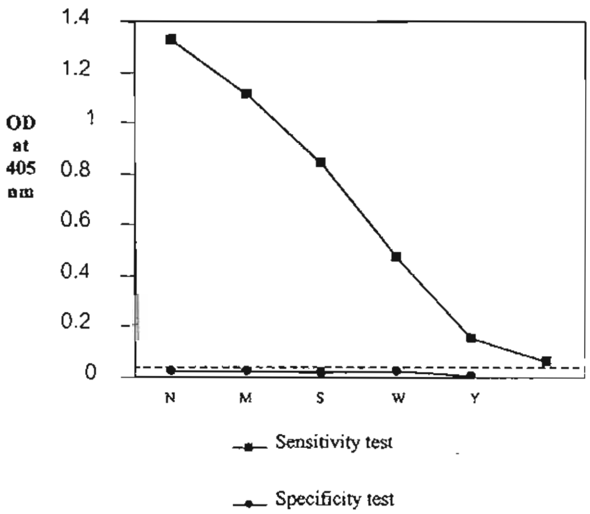

Fig. 2. Graphical illustration of sensitivity and specificity of the PCR-ELISA. The absorbance of the color products were measured at 405 nm using ELISA photometric measurement apparatus. The upper line (–■–) shows the sensitivity for detection of HPV DNA at the concentration of 1000, 100, 10, 1, 0·1, 0·01 fg, respectively. The lower line (–●–) demonstrates the specificity. The dotted line represents the mean value of negative control. The letters N, W, Y, M, and S represent negative control, monodon baculovirus (MBV) white-spot syndrome virus (WSV), shrimp (S), and yellow-head virus (YHV) nucleic acids, respectively.

Detection of HPV in crude sample in comparison to histological examination

The reliability of detection using PCR-ELISA assay was compared directly to histological examination which used as a gold standard method. Hepatopancras of infected and healthy individual shrimps was divided into two parts for histological examination (H&E staining) and PCR-ELISA assay. Crude DNA templates were simple prepared and analyzed by PCR-ELISA. A total of 12 samples examined, 10 samples were positive by PCR-ELISA assay and 9 samples were positive by H&E staining. This result (Table 1) show the ability of detection by both methods with 92% agreement and 100% efficiency of detection by PCR-ELISA.

Detection of HPV in carrier shrimps by conventional PCR and PCR-ELISA

To determine the efficiency of PCR-ELISA assay for detection of HPV in carrier shrimp in field, a total of 87 hepatopancreases of shrimp samples collected from ponds in the central and southern part of Thailand, were analyzed by PCR-ELISA in comparing to conventional PCR. There were two set of samples, the first set containing 42 samples which were HPV negative detected by H&E staining and the second set composed of 45 hepatopancreases derived from stunted shrimp. The results in Table 2 shows that 65 samples gave positive detection by PCR-ELISA but only 53 samples by conventional PCR. Twenty-six out of 42 (62%) of the first set of samples were tested positive by PCR-ELISA. It also found that 87% of the stunted shrimps were infected with HPV.

DISCUSSION AND CONCLUSION

PCR-ELISA developed in this study is an alternative approach to diagnose HPV infection in shrimp and carriers. The detection method is simpler than both histological techniques, as tissues must be subsequently processed and conventional PCR, in which the amplified product needs to be electrophoretically examined. The protocol does not require hazardous substances such as ethidium bromide. In terms of sensitivity, the lowest amount of purified HPV DNA detected by this technique was 0·01 fg (approximately three viral particles) which was 10 and 100 times more sensitive than that of the Southern hybridization and conventional PCR, respectively.

For filed application, PCR-ELISA also showed more sensitive than conventional PCR and histological examination. Several shrimp samples which gave negative detection by H&E staining but positive by PCR-ELISA, this implied that these shrimps were the carrier of the virus. Therefore, the PCR-ELISA may be a suitable technique for detecting HPV infection in carriers and broodstock.

At present, the PCR -ELISA techniques is available commercially in a microtiter plate format (Boehringer Mannheim), allowing flexibility of the detection, as positive HPV signals can be directly visualized when qualitative results are sufficient. On the other hand, quantitative results can be gotten using a microtiter plate reader when detailed data are needed. Reduction of the hybridization period to only 30 min, as illustrated in this study, gave a similar result to that using a standard hybridization time so that modified PCR-ELISA offers a consistent diagnostic result which is more rapid than other HPV detection methods. The disadvantage of this technique is that it is more expensive than the conventional PCR assay, but this

Table 1. Comparison of PCR-ELISA and histological examination (H&E staining) for detection of 12 hepatopancreases samples tested

PCR-ELISA+ H&E+	PCR-ELISA+ H&E−	PCR-ELISA− H&E+	PCR-ELISA− H&E−	Sensitivity	Specificity
9	1	0	2	100%	8·33%

Table 2. The detection HPV infection from 87 carrier shrimps by conventional PCR and PCR-ELISA

Shrimp samples	PCR-ELISA+ PCR+	PCR-ELISA+ PCR−	PCR-ELISA− PCR+	PCR-ELISA− PCR−
HPV negative by H&E	15	11	0	16
Stunted characteristic	38	1	0	6

disadvantage is mitigated by its convenience and sensitivity.

ACKNOWLEDGEMENTS

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5.3.3

Kiatpathomchai W.,Boonsaeng V.,Tassanakajon A.,**Wongteerasupaya** C., Jitrapakdee S., Panyim, S. A non – stop , single -tabe, Semi-nested PCR technique for grading the severit of white spot syndrome virus infections in Penaues monodon . *Disease of Aquatic Organisms* Vol 47 Dec. 2001,Pages 235- 239

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NOTE

A non-stop, single-tube, semi-nested PCR technique for grading the severity of white spot syndrome virus infections in *Penaeus monodon*

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ABSTRACT: A single-tube, non-stop, semi-nested polymerase chain reaction (PCR) technique was developed for simultaneous detection and severity grading of white spot syndrome virus (WSSV) infections in the black tiger shrimp *Penaeus monodon*. The test uses 1 sense primer and 3 antisense primers that produce up to 3 PCR products (1100, 526 and 250 base pairs [bp]) depending upon the severity of infection. Specifically, heavy infections ($\geq 2 \times 10^4$ viral particles) of WSSV produce all 3 fragments, while moderate infections (around 2×10^3 viral particles) produce 2 (526 and 250 bp) and light infections (20 to 200 viral particles) produce 1 (250 bp). In addition, the technique uses internal control primers that yield a shrimp characteristic fragment for non-infected samples and samples with a low quantity of viral target in order to assure integrity and reproducibility of the PCR assays. The non-stop, single-tube, semi-nested PCR technique is simple and convenient and can detect as little as 5 fg WSSV DNA (20 viral particles) in crude extracts of postlarval samples or extracts of pleopods and haemolymph from larger shrimp.

KEY WORDS: White spot syndrome virus · WSSV · Single-tube nested PCR · *Penaeus monodon*

White spot syndrome virus (WSSV) is a viral disease that affects most of the commercially cultivated marine shrimp species, not just in Asia but globally (Wongteerasupaya et al. 1996, Flegel 1997, Lu et al. 1997, Lo et al. 1999, van Hulten et al. 2000). In Thailand, it was first reported as an accidental infection in laboratory-reared shrimp in early 1994 (Wongteerasupaya et al. 1995) although a virus with similar morphology had been reported earlier from farmed specimens of *Penaeus japonicus* in Japan (Nakano et al. 1994, Takahashi et al. 1994) and *P. japonicus*, *P. monodon* and *P. penicillatus* in Taiwan (Chen & Kou 1994).

WSSV is an enveloped, rod-shaped virus, containing double-stranded DNA (Wang et al. 1995, Wongteerasupaya et al. 1995). Contrary to initial speculation (Wongteerasupaya et al. 1995), it is unrelated to baculoviruses (Tsai et al. 2000, van Hulten et al. 2001) and it has been recommended that it be included in a new family tentatively called *Whispoviridae* or *Nimaviridae* (van Hulten et al. 2000, 2001). For transmission electron microscopy, negative staining from hemolymph samples revealed that intact virions were 121 ± 9 nm in width at the widest point and 276 ± 26 nm in length, excluding a multifilament appendage that was often seen attached at the narrow end (Wongteerasupaya et al. 1995). Acutely infected shrimp often show white spots of 0.5 to several mm in diameter in the cuticle (which are abnormal deposits of calcium) and sometimes also a pink to reddish-brown coloration due to expansion of sub-cuticular chromatophores (Lightner 1996).

WSSV can be detected by histological and molecular techniques, of which PCR is the most sensitive. Currently reported PCR techniques for WSSV detection use either a conventional amplification with a single sense/antisense primer set (Lo et al. 1996a, Kanchanaphum et al. 1998, Withyachumnarnkul 1999) or a nested amplification (Lo et al. 1996b, 1998). Nested PCR provides an increased level of sensitivity compared with conventional single primer-pair PCR. Real-time PCR is more sensitive and has been reported for the quantification of infectious hypodermal and hema-

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topoietic necrosis virus in infected penaeid shrimp (Tang & Lightner 2001) but not for WSSV.

One of the major drawbacks with 2-step nested PCR is the risk of introducing contamination when a second PCR reaction is initiated using a portion of the mixture from the first reaction vial. This problem can be circumvented by designing a 1-tube, non-stop, semi-nested PCR where 2 or 3 primer pairs with different melting points are simultaneously added with all reaction components and the test sample in a single tube (Rolfs et al. 1992). The aim of this work was to develop a semi-quantitative PCR detection method for WSSV that can be carried out as a single-tube, non-stop, semi-nested PCR amplification.

Methods and results. WSSV was isolated from experimentally infected juvenile shrimp *Penaeus monodon*, and the WSSV DNA was extracted from purified virions using phenol-chloroform extraction as described by Wongteerasupaya et al. (1995).

Twenty *Penaeus monodon* postlarvae or 1 pleopod stored in ethanol were homogenized with a mixture of 200 µl of 0.025 N NaOH and 0.0125% sodium dodecyl sulphate in a 1.5 ml microcentrifuge tube, followed by incubation in boiling water for 5 min before immediate incubation on ice. After a brief centrifugation at 10000 rpm in a benchtop microcentrifuge, 5 µl of the supernatant was used as the template for PCR amplification.

Ten microliters of hemolymph from *Penaeus monodon* was mixed with 20 µl of 0.005 N NaOH, incubated at 95°C for 5 min then transferred to ice for 5 min before brief centrifugation. Five microliters of the supernatant was used as the template for PCR amplification.

Using primer analysis software (Oligo 4.0s, National Biosciences, Inc., Plymouth, MN, USA), 1 sense primer (F1) and 3 antisense primers (R1, R2 and R3) were designed from the nucleotide sequence of a 4.2 kb WSSV clone (Wongteerasupaya et al. 1995). The sequences of these primers are shown in Table 1. The F1 and R1 primers were used as external primers to generate a primary PCR product of 1100 base pairs (bp) while R2 and R3 primers were used as internal primers along with F1 to generate nested PCR products of 526 bp and 250 bp, respectively, as shown in Fig. 1.

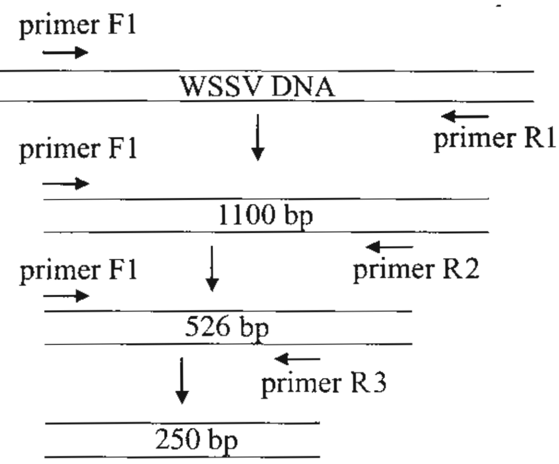

Fig. 1. Diagram showing the products of the non-stop, single-tube, semi-nested polymerase chain reaction for detection of WSSV. bp: Base pairs

Primers for the shrimp-specific PCR product comprised primers PMN1 and PMN2 (Table 1) designed from the actin gene of *Penaeus monodon* (Boonyawan 1998). They produced a fragment of 143 bp.

The 50 µl PCR reaction contained 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 1.5 mM $MgCl_2$, 0.2 mM each of deoxy (d) ATP, dCTP, dGTP and dTTP, 1 µM F1, 0.2 µM R1, 0.3 µM R2, 0.5 µM R3, and 0.8 µM each of PMN1 and PMN2. The PCR reaction was initiated by heating the mixture to 93°C for 5 min followed by addition of 6 U of *Taq* polymerase at 75°C. This was followed by 3 successive cycling protocols of (1) 5 cycles of 93°C for 20 s, 70°C for 20 s and 72°C for 20 s, followed by (2) 20 cycles of 93°C for 20 s, 55°C for 20 s and 72°C for 20 s, followed by (3) 25 cycles of 93°C for 20 s, 50°C for 20 s and 72°C 20 s. Following PCR, 10 µl of the reaction solution was examined following electrophoresis through a 2% w/v agarose gel and ethidium bromide staining.

Table 1. Sequences of the primers used. These were designed based on the sequence of a 4.2 kb cloned fragment derived from purified WSSV and from the sequence of the actin gene of *Penaeus monodon*

Primer	Sequence	Tm (°C)	Target DNA	Product size (bp)
F1	5'AGAGCCCGAATAGTGTTTCCTCAGC 3'	76	WSSV DNA	–
R1	5'CAGGCAATATAGCCCGTTTGGG 3'	68	WSSV DNA	1100
R2	5'ATTGCCAATGTGACTAAGCGG 3'	62	WSSV DNA	526
R3	5'AACACAGCTAACCTTTATGAG 3'	58	WSSV DNA	250
PMN1	5'CATGATTATTTTGTATATATTATCG 3'	60	Shrimp DNA	143
PMN2	5'CAAGTGCTTCTAAGGATACTG 3'	60	Shrimp DNA	

To determine the sensitivity of the non-stop, semi-nested PCR assay, purified WSSV DNA was serially diluted in 10-fold steps from 50 pg to 0.5 fg and added to normal homogenized postlarval shrimp (i.e., free of WSSV as determined by PCR assay) for use as a template in 1-step nested PCR. By agarose gel electrophoresis, non-stop, semi-nested PCR amplification of these templates produced from 1 to 3 PCR products in a manner dependent upon the quantity of WSSV

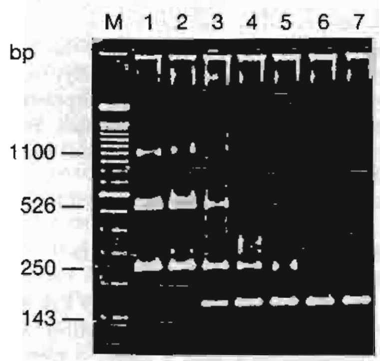

Fig. 2. Sensitivity of the non-stop, single-tube, semi-nested PCR for the detection of WSSV. The PCR products were amplified from purified WSSV DNA serially diluted (1:10) in the range of 50 pg to 0.5 fg, added to a homogenate of shrimp postlarvae and extracted as a PCR template (Lanes 1 to 6, respectively). Lane 7 shows the PCR product from a template crude extract of normal postlarvae without added WSSV DNA. The highest dilution still giving a positive reaction was 5 fg of WSSV DNA in crude postlarval homogenate (Lane 5). Lane M is a 100 bp DNA ladder

added to the homogenates (Fig. 2). Quantities of WSSV DNA at 5 pg (2×10^4 viral particles) and greater gave 3 PCR products of 1100, 526 and 250 bp (Fig. 2, Lanes 1 and 2). A moderate concentration of WSSV DNA, around 500 fg (2×10^3 viral particles), gave 2 products of 526 and 250 bp (Fig. 2, Lane 3) and a low concentration or 5 to 50 fg (20 to 200 viral particles) gave only 1 product of 250 bp (Fig. 2, Lanes 4 and 5). The limit of detection was 5 fg or approximately 20 viral particles

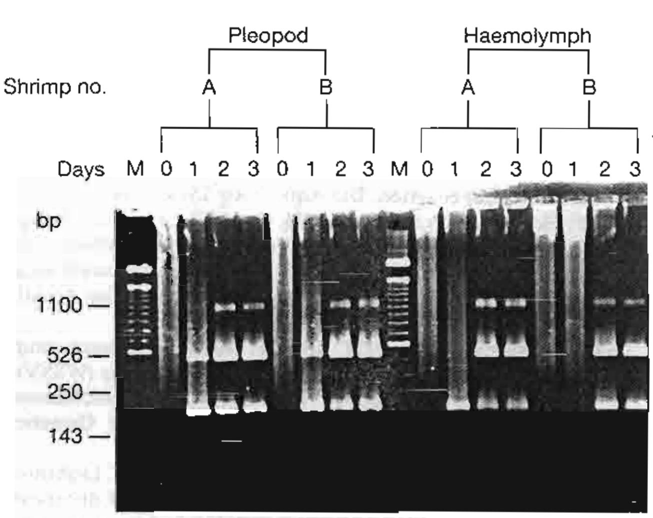

Fig. 3. Ethidium bromide staining of non-stop, single-tube, semi-nested PCR products derived from pleopods and haemolymph of 2 juvenile shrimp (A and B) 1, 2 and 3 days after injection with WSSV (Lanes 1, 2 and 3, respectively). Lane 0 is an extract derived before injection of the virus. Lane M is a 100 bp DNA ladder

(Fig. 2, Lane 5). In addition, a shrimp-specific product at 143 bp was observed for the samples when no or low quantities of WSSV DNA were added (Fig. 2, Lanes 3, 4, 5, 6 and 7). When the concentration of the WSSV target was in the range of approximately 500 fg or more, the shrimp-specific, internal control product was not observed.

Detection of WSSV infection in experimentally infected shrimp was tested using tissue and hemolymph derived from 2 juvenile shrimp experimentally infected with WSSV and reared in an aquarium. Hemolymph (10 µl) and 1 pleopod were removed daily for testing using the non-stop, semi-nested technique. At 1 d post injection, 2 PCR products (526 and 250 bp) were seen from the pleopods of both shrimp (A & B in Fig. 3) and this increased to 3 fragments (1100, 526 and 250 bp) at Days 2 and 3. With hemolymph from both shrimp, only 1 fragment (250 bp) was observed at Day 1 and 3 fragments at Days 2 and 3. The 143 bp fragment specific for shrimp was seen only in the samples taken at time zero, before WSSV injection.

To test that the non-stop, semi-nested PCR protocol could detect WSSV in field samples of postlarvae, 20 batches were stored in 95% ethanol and delivered to the laboratory by shrimp farmers. These were typical of the samples usually supplied to commercial test laboratories in Thailand. The postlarvae were removed from alcohol, touched on a paper towel to remove excess alcohol and then processed (i.e., homogenized and extracted as described above) before being tested. A range of results was obtained from 0 to 3 WSSV characteristic fragments. The shrimp-specific marker fragment of 143 bp was seen only with samples that gave either no fragments for WSSV or patterns that indicated low to medium quantities of WSSV.

Discussion. Nested PCR reactions are now used widely in the detection of a number of viruses such as hepatitis C virus (Svoboda-Newman et al. 1996), bovine herpesvirus (Ashbaugh et al. 1997) and fish rhabdoviruses (Miller et al. 1998). They are also used routinely for detection of penaeid shrimp viruses including WSSV (Lo et al. 1996b), monodon baculovirus (Belcher & Young 1998), gill-associated virus and lymphoid organ virus (Cowley et al. 2000). However, most of these currently described nested PCR methods use conventional steps where a portion of the PCR reaction from the first step is transferred to a second PCR reaction tube for the second (nested) amplification. There is a high contamination risk at this transfer step. By placing all of the reagents for the nested reaction in a single tube and using a 3-phase amplification program, we have attempted to circumvent this potential problem. To our knowledge, the details of a such a semi-quantitative, non-stop, semi-nested PCR detection method have not yet been described for any virus of penaeid shrimp.

With respect to sensitivity, we estimate that the lowest detection level of 5 fg of WSSV DNA is equivalent to approximately 20 viral particles based on the estimated length of the total WSSV genome being in the range of 300 kb (Yang et al. 1997) and assuming only a single copy of the target sequence for the primers. This estimated level of sensitivity is comparable with that of the 2-step nested PCR technique reported by Lo et al. (1998). However, our method is an improvement because it is an uninterrupted, single-tube reaction with reduced risks for contamination. A commercial application of the method of Lo et al. (1998) (Farming IntelliGene Tech. Co., Taipei, Taiwan) uses a single PCR tube but it must be opened after completion of the first PCR step for addition of a reagent premix containing a pair of internal primers for the second PCR step. These tube openings and transfers create additional risks of contamination. One very important advantage of our method is its ability to grade the severity of viral infections to 3 levels. Commercial products that achieve a similar grading are available but information regarding the primers and reagents used are proprietary. Grading of infections has been reported to be important for the management of WSSV in culture systems (Lo et al. 1998, Peng et al. in press), and our technique should provide an open alternative to commercial systems currently offered for the purpose.

Tan et al. (2001) have reported a method for WSSV quantification based on competitive PCR. This may be useful when precise measurements of WSSV quantity are required. However, the method was not suitable for detection of very low viral loads, as is required when shrimp farmers screen potential WSSV carriers and test postlarvae before stocking shrimp ponds. Our method would be more suitable for such routine assays where precise quantification of viral load is not critical.

Various protocols of sample preparation for WSSV detection have been developed using cetyltrimethylammonium bromide followed by chloroform extraction and precipitation of nucleic acids with 2 volumes of absolute ethanol (Lo et al. 1996a) or DNAzol® Reagent (Gibco Life Technologies; Magbanua et al. 2000). These methods are complicated, time consuming, and risk contamination and loss of DNA during the extraction process. We have successfully circumvented the need for this by using a simplified, crude extraction procedure that gives satisfactory results under the conditions of our assay. Using the protocol described in this paper, we have not found evidence of PCR inhibitors since we always obtain either PCR amplicons of expected sizes or the internal control amplicon in test samples. On the other hand, we cannot exclude the possibility that some partial inhibitors might reduce the sensitivity of the assay for WSSV.

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5.3.4

Chainarong Wongteerasupaya , Studies of genelic variatian Among geographic isolates of white spot syndrome virus using enveloped protein VP28 Sequence งานวิจัยด้านอณูชีววิทยา กลุ่มเมธีวิจัยอาวุโสสกว.14 กันยายน 2544 หน้า 3/1-5 ศ.ดร.สกล พันธุ์ยิ้ม

Studies of genetic variations among geographic isolates of white spot syndrome virus using enveloped protein VP28 sequence.

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Abstract

White spot syndrome virus (WSSV) is a major viral disease agent distributed in Thailand where panaeid shrimp are cultured. Six isolated of WSSV from Surathanee, Chumporn and Chachaoengshao were compared by analysing the viral envelope protein VP28 sequence. No distinctive difference among the WSSV isolates from penaeid shrimp were detected. The results indicate that some viral protein components of WSSV from different geographic regions share a vary high degree of homology.

Introduction

White spot syndrome virus (WSSV) is a major viral disease agent in shrimp culture. Electron microscopy studies showed that the virions are enveloped and have a bacilliform shaped of 275 nm in length and 120 nm in width with a tail-like appendage of one end (Wongteerasupaya et al.,1995). Nucleocapsides, which have lost their envelope, has a cross-hatched appearance and a size of 300 x 70 nm (Wongteerasupaya et al.,1995). This viral morphology, its nuclear localization, and its morphogenesis are reminiscent of baculoviruses in insects (Durand et al.,1997). The phylogenetic analysis of ribonucleotide reductase gene indicated that WSSV belongs to the eukaryotic branch of an unrooted parsimonious tree and suggest to be a number of a new genus (Whispovirus) within the baculoviridae or a number of an entirely new virus family (Van Hulten et al.,1999). Other genes that are highly conserved in related virus are those coding for viral capsid and envelope protein (Morphy et al.,1995). This two WSSV virion protein showed no homology to known baculovirus protein in databases.

The virus has a wide host range among crustaceans (Flegel,1997) and distinctive clinical signs in penaeid shrimp. There is little genetic variation among WSSV isolates from around the world using combinning the method of restrinction analysis and southern blot hybridization. In this paper, we study the relationships between WSSV isolates based on sequence analysis of viral enveloped protein VP28 gene.

Material and Method

The virions were isolated from infected *penaeus monodon* shrimp from Thailand.Infected tissue was homogenized in TN buffer (20mM Tris-HCl,400 mM NaCl,pH 7.4) after centrifugation at 1700 g for 10 min,the viral DNA was isolated by treament with proteinase K (0.2mg/ml) and sarkosyl(1%) at 45°C for 2-4 hr. followed by phenol-chloroform extraction.

Primer and PCR reaction

PCR primer of VP28 coding regions

VP28F	ATGGATCTTTCTTTCACTCTC
VP28R	TTACTCGGTCTCAGTGCCAG

PCR primer of nVP28 for non coding regions

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nVP28F    ATTACGGTTACGAAAATTCC
nVP28R    AGAGTGAAAGAAAGATCCAT
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The PCR reaction,10 ng DNA and 30 pmol each primer were added to a reaction mixture comprising 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2mM $MgCl_2$,200μM each dATP,dTTP,dCTP and dGTP, 25U Taq Pwo DNA polymerase (Gibco-BRL). The mixture was incubated in a thermal cycler using 30 cycle of 94°C for 30s, 52°C for 30s,and 72°C for 1 min and a final elongation step of 72°C for 10 min. PCR products 608 bp of VP28 coding region and 453 bp of noncoding region were purified using a QIA quick™ column(QIAGEN).

Sequence analysis

The 608 bp PCR products of VP28 coding region from 4 WSSV isolates and 453 bp PCR product of VP28 noncoding region from 6 WSSV isolates were sequence using thermo Sequence™ (Amersham) and Automated ABI model 377 Sequencing system (Applied biosystem Inc.). Sequence alignmemt and estimation of sequence identity were conducted using Clustal W software.

Results and discussion

The relationship between the WSSV isolates were determined by sequence comparison of VP28 coding gene (Fig 1.) and VP28 noncoding gene (Fig 2). The results indicate that WSSV isolates of these studies share a vary high degree of homology. Sequence analysis of WSSV from various region in Australia, India, and the Americans will be required to determined the extent of molecular diversity among WSSV isolates.

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Sur2vp28    CTCGCCATCACTGCTGTGATTGCTGTATTTATTGTGATTTTTAGGTATCACAA
Sur7vp28    CTCGCCATCACTGCTGTGATTGCTGTATTTATTGTGATTTTTAGGTATCACAA
Chu1vp28    CTCGCCATCACTGCTGTGATTGCTGTATTTATTGTGATTTTTAGGTATCACAA
Chu2vp28    CTCGCCATCACTGCTGTGATTGCTGTATTTATTGTGATTTTTAGGTATCACAA
Vp28        CTCGCCATCACTGCTGTGATTGCTGTATTTATTGTGATTTTTAGGTATCACAA

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Sur2vp28    CACTGTGACCAAGACCATCGAAACCCACACAGACAATATCGAGACAAACATGG
Sur7vp28    CACTGTGACCAAGACCATCGAAACCCACACAGACAATATCGAGACAAACATGG
Chu1vp28    CACTGTGACCAAGACCATCGAAACCCACACAGACAATATCGAGACAAACATGG
Chu2vp28    CACTGTGACCAAGACCATCGAAACCCACACAGACAATATCGAGACAAACATGG
Vp28        CACTGTGACCAAGACCATCGAAACCCACACAGACAATATCGAGACAAACATGG

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Sur2vp28    ATGAAAACCTCCGCATTCCTGTGACTGCTGAGGTTGGATCAGGCTACTTCAAG
Sur7vp28    ATGAAAACCTCCGCATTCCTGTGACTGCTGAGGTTGGATCAGGCTACTTCAAG
Chu1vp28    ATGAAAACCTCCGCATTCCTGTGACTGCTGAGGTTGGATCAGGCTACTTCAAG
Chu2vp28    ATGAAAACCTCCGCATTCCTGTGACTGCTGAGGTTGGATCAGGCTACTTCAAG
Vp28        ATGAAAACCTCCGCATTCCTGTGACTGCTGAGGTTGGATCAGGCTACTTCAAG

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Sur2vp28    ATGACTGATGTGTCCTTTGACAGCGACACCTTGGGCAAAATCAAGATCCGCAA
Sur7vp28    ATGACTGATGTGTCCTTTGACAGCGACACCTTGGGCAAAATCAAGATCCGCAA
Chu1vp28    ATGACTGATGTGTCCTTTGACAGCGACACCTTGGGCAAAATCAAGATCCGCAA
Chu2vp28    ATGACTGATGTGTCCTTTGACAGCGACACCTTGGGCAAAATCAAGATCCGCAA
Vp28        ATGACTGATGTGTCCTTTGACAGCGACACCTTGGGCAAAATCAAGATCCGCAA
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Sur2vp28      TGGAAAGTCTGATGCACAGATGAAGGAAGAAGATGCGGATCTTGTCATCA
Sur7vp28      TGGAAAGTCTGATGCACAGATGAAGGAAGAAGATGCGGATCTTGTCATCA
Chu1vp28      TGGAAAGTCTGATGCACAGATGAAGGAAGAAGATGCGGATCTTGTCATCA
Chu2vp28      TGGAAAGTCTGATGCACAGATGAAGGAAGAAGATGCGGATCTTGTCATCA
Vp28          TGGAAAGTCTGATGCACAGATGAAGGAAGAAGATGCGGATCTTGTCATCA
              - - - - - - - - - - - - - - - - - - - - - - - - -

Sur2vp28      CTCCCGTGGAGGGCCGAGCACTCGAAGTGACTGTGGGGCAGAATCTCACC
Sur7vp28      CTCCCGTGGAGGGCCGAGCACTCGAAGTGACTGTGGGGCAGAATCTCACC
Chu1vp28      CTCCCGTGGAGGGCCGAGCACTCGAAGTGACTGTGGGGCAGAATCTCACC
Chu2vp28      CTCCCGTGGAGGGCCGAGCACTCGAAGTGACTGTGGGGCAGAATCTCACC
Vp28          CTCCCGTGGAGGGCCGAGCACTCGAAGTGACTGTGGGGCAGAATCTCACC
              - - - - - - - - - - - - - - - - - - - - - - - - -

Sur2vp28      TTTGAGGGAACATTCAAGGTGTGGAACAACACATCAAGAAAGATCAACAT
Sur7vp28      TTTGAGGGAACATTCAAGGTGTGGAACAACACATCAAGAAAGATCAACAT
Chu1vp28      TTTGAGGGAACATTCAAGGTGTGGAACAACACATCAAGAAAGATCAACAT
Chu2vp28      TTTGAGGGAACATTCAAGGTGTGGAACAACACATCAAGAAAGATCAACAT
Vp28          TTTGAGGGAACATTCAAGGTGTGGAACAACACATCAAGAAAGATCAACAT
              - - - - - - - - - - - - - - - - - - - - - - - - -

Sur2vp28      CACTGGTATGCAGATGGTGCCAAAGATTAACCCATCAAAGGCCTTTGTCG
Sur7vp28      CACTGGTATGCAGATGGTGCCAAAGATTAACCCATCAAAGGCCTTTGTCG
Chu1vp28      CACTGGTATGCAGATGGTGCCAAAGATTAACCCATCAAAGGCCTTTGTCG
Chu2vp28      CACTGGTATGCAGATGGTGCCAAAGATTAACCCATCAAAGGCCTTTGTCG
Vp28          CACTGGTATGCAGATGGTGCCAAAGATTAACCCATCAAAGGCCTTTGTCG
              - - - - - - - - - - - - - - - - - - - - - - - - -

Sur2vp28      GTAGCTCCAACACCTCCTCCTTCACCCCCGTCTNATTGATGAGGATGAA
Sur7vp28      GTAGCTNCAACACCTCCTCCTTCACCCCCGTCTTTATTGATGAGGATGAA
Chu1vp28      GTAGCTCCAACACCTCCTCCTTCACCCCCGTCTNATTGATGAGGATGAA
Chu2vp28      GTAGCTCCAACACCTCCTCCTTCACCCCCGTCTNATTGATGAGGATGAA
Vp28          GTAGCTCCAACACCTCCTCCTTCACCCCCGTCTCTATTGATGAGGATGAA
                    *                          *
              - - -   - - - - - - - - - - - - - - - - - - - - -

Sur2vp28      GTTGGCACCTTTGTGTGTGGTACCACCTTTGGCGCACCAATTGCAGCTCC
Sur7vp28      GTTGGCACCTTTGTGTGTGGTACCACCTTTGGCGCACCAATTGCAGCTCC
Chu1vp28      GTTGGCACCTTTGTGTGTGGTACCACCTTTGGCGCACCAATTGCAGCTCC
Chu2vp28      GTTGGCACCTTTGTGTGTGGTACCACCTTTGGCGCACCAATTGCAGCTCC
Vp28          GTTGGCACCTTTGTGTGTGGTACCACCTTTGGCGCACCAATTGCAGCTCC
              - - - - - - - - - - - - - - - - - - - - - - - - -

Sur2vp28      GCCGGTGGAAATCTTTTCGACATGTAC
Sur7vp28      GCCGGTGGAAATCTTTTCGACATGTAC
Chu1vp28      GCCGGTGGAAATCTTTTCGACATGTAC
Chu2vp28      GCCGGTGGAAATCTTTTCGACATGTAC
Vp28          GCCGGTGGAAATCTTTTCGACATGTAC
              - - - - - - - - - - - - - -
```

Fig.1 Multiple Sequence Alingment of VP28 coding gene of WSSV
Sur= Surathanee Chu= Chumporn
Ref= a reference VP28 coding sequence reported by van Hulten et al 2000

```
Sur2nvp28    AACTAAAAAAATTCAATACTGGCACTGGATGATTGGGGTTCATTTTATTT
Sur7nvp28    AACTAAAAAAATTCAATACTGGCACTGGATGATTGGGGTTCATTTTATTT
Chu1nvp28    AACTAAAAAAATTCAATACTGGCACTGGATGATTGGGGTTCATTTTATTT
Chu2nvp28    AACTAAAAAAATTCAATACTGGCACTGGATGATTGGGGTTCATTTTATTT
Pad1nvp28    AACTAAAAAAATTCAATACTGGCACTGGATGATTGGGGTTCATTTTATTT
Pad2nvp28    AACTAAAAAAATTCAATACTGGCACTGGATGATTGGGGTTCATTTTATTT
vp28         AACTAAAAAAATTCAATACTGGCACTGGATGATTGGGGTTCATTTTATTT
             - - - - - - - - - - - - - - - - - - - - - - - - -

Sur2nvp28    ATTTTATTAATCATCGGAATTTGGGGTTTATTTAGATCTCGAAATGCAAC
Sur7nvp28    ATTTTATTAATCATCGGAATTTGGGGTTTATTTAGATCTCGAAATGCAAC
Chu1nvp28    ATTTTATTAATCATCGGAATTTGGGGTTTATTTAGATCTCGAAATGCAAC
Chu2nvp28    ATTTTATTAATCATCGGAATTTGGGGTTTATTTAGATCTCGAAATGCAAC
Pad1nvp28    ATTTTATTAATCATCGGAATTTGGGGTTTATTTAGATCTCGAAATGCAAC
Pad2nvp28    ATTTTATTAATCATCGGAATTTGGGGTTTATTTAGATCTCGAAATGCAAC
Nvp28        ATTTTATTAATCATCGGAATTTGGGGTTTATTTAGATCTCGAAATGCAAC
             - - - - - - - - - - - - - - - - - - - - - - - - -

Sur2nvp28    CACCCAAGAGANCAAAACTTCTTCCCCAACAATCTCCTCGACCCCAACTA
Sur7nvp28    CACCCAAGAGAGCAAAACTTCTTCCCCAACAATCTCCTCGACCCCAACTA
Chu1nvp28    CACCCAAGAGANCAAAACTTCTTCCCCAACAATCTCCTCGACCCCAACTA
Chu2nvp28    CACCCAAGAGAGCAAAACTTCTTCCCCAACAATCTCCTCGACCCCAACTA
Pad1nvp28    CACCCAAGAGAGCAAAACTTCTTCCCCAACAATCTCCTCGACCCCAACTA
Pad2nvp28    CACCCAAGAGAGCAAAACTTCTTCCCCAACAATCTCCTCGACCCCAACTA
Nvp28        CACCCAAGAGAGCAAAACTTCTTCCCCAACAATCTCCTCGACCCCAACTA
             - - - - - -*- - - - - - - - - - - - - - - - - - -

Sur2nvp28    CATATTCTGGCAGCTCAACCAGCAGGGGTCCAGGTTCTGGATCTGGAAAC
Sur7nvp28    CATATTCTGGCAGCTCAACCAGCAGGGGTCCAGGTTCTGGATCTGGAAAC
Chu1nvp28    CATATTCTGGCAGCTCAACCAGCAGGGGTCCAGGTTCTGGATCTGGAAAC
Chu2nvp28    CATATTCTGGCAGCTCAACCAGCAGGGGTCCAGGTTCTGGATCTGGAAAC
Pad1nvp28    CATATTCTGGCAGCTCAACCAGCAGGGGTCCAGGTTCTGGATCTGGAAAC
Pad2nvp28    CATATTCTGGCAGCTCAACCAGCAGGGGTCCAGGTTCTGGATCTGGAAAC
Nvp28        CATATTCTGGCAGCTCAACCAGCAGGGGTCCAGGTTCTGGATCTGGAAAC
             - - - - - - - - - - - - - - - - - - - - - - - - -

Sur2nvp28    AAACCCAAAGATGACACATCCGTTGAAGGAATAGACCCTGGCTTACTGTA
Sur7nvp28    AAACCCAAAGATGACACATCCGTTGAAGGAATAGACCCTGGCTTACTGTA
Chu1nvp28    AAACCCAAAGATGACACATCCGTTGAAGGAATAGACCCTGGCTTACTGTA
Chu2nvp28    AAACCCAAAGATGACACATCCGTTGAAGGAATAGACCCTGGCTTACTGTA
Pad1nvp28    AAACCCAAAGATGACACATCCGTTGAAGGAATAGACCCTGGCTTACTGTA
Pad2nvp28    AAACCCAAAGATGACACATCCGTTGAAGGAATAGACCCTGGCTTACTGTA
Nvp28        AAACCCAAAGATGACACATCCGTTGAAGGAATAGACCCTGGCTTACTGTA
             - - - - - - - - - - - - - - - - - - - - - - - - -

Sur2nvp28    ACAGAAAAAAAGAGTAAAAGGCGACAGCTCGCTTGCCAATTGTCCTGTTAC
Sur7nvp28    ACAGAAAAAAAGAGTAAAAGGCGACAGCTCGCTTGCCAATTGTCCTGTTAC
Chu1nvp28    ACAGAAAAAAAGAGTAAAAGGCGACAGCTCGCTTGCCAATTGTCCTGTTAC
Chu2nvp28    ACAGAAAAAAAGAGTAAAAGGCGACAGCTCGCTTGCCAATTGTCCTGTTAC
Pad1nvp28    ACAGAAAAAAAGAGTAAAAGGCGACAGCTCGCTTGCCAATTGTCCTGTTAC
Pad2nvp28    ACAGAAAAAAAGAGTAAAAGGCGACAGCTCGCTTGCCAATTGTCCTGTTAC
Nvp28        ACAGAAAAAAAGAGTAAAAGGCGACAGCTCGCTTGCCAATTGTCCTGTTAC
             - - - - - - - - - - - - - - - - - - - - - - - - -

Sur2nvp28    GTACTCTGTGGTTTCACGAGGTTGTCATCACCAAAGGTAACCTTTTTTTT
Sur7nvp28    GTACTCTGTGGTTTCACGAGGTTGTCATCACCAAAGGTAACCTTTTTTTT
Chu1nvp28    GTACTCTGTGGTTTCACGAGGTTGTCATCACCAAAGGTAACCTTTTTTTT
Chu2nvp28    GTACTCTGTGGTTTCACGAGGTTGTCATCACCAAAGGTAACCTTTTTTTT
Pad1nvp28    GTACTCTGTGGTTTCACGAGGTTGTCATCACCAAAGGTAACCTTTTTTTT
Pad2nvp28    GTACTCTGTGGTTTCACGAGGTTGTCATCACCAAAGGTAACCTTTTTTTT
Nvp28        GTACTCTGTGGTTTCACGAGGTTGTCATCACCAAAGGTAACCTTTTTTTT
             - - - - - - - - - - - - - - - - - - - - - - - - -
```

```
Sur2nvp28    TGTCCTCGCCGACAAAACGACATCTTAATAACCAAGCAACGTTCGATAAA
Sur7nvp28    TGTCCTCGCCGACAAAACGACATCTTAATAACCAAGCAACGTTCGATAAA
Chu1nvp28    TGTCCTCGCCGACAAAACGACATCTTAATAACCAAGCAACGTTCGATAAA
Chu2nvp28    TGTCCTCGCCGACAAAACGACATCTTAATAACCAAGCAACGTTCGATAAA
Pad1nvp28    TGTCCTCGCCGACAAAACGACATCTTAATAACCAAGCAACGTTCGATAAA
Pad2nvp28    TGTCCTCGCCGACAAAACGACATCTTAATAACCAAGCAACGTTCGATAAA
Nvp28        TGTCCTCGCCGACAAAACGACATCTTAATAACCAAGCAACGTTCGATAAA
             - - - - - - - - - - - - - - - - - - - - - - - - -
```

Fig.2 Multiple Sequence Alingment VP28 noncoding gene of WSSV
Sur= Surathanee Chu= Chumporn Cha=Chachaoengshao
Ref= a reference VP28 noncoding sequence reported by van Hulten et al 2000

Acknowledgments

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5.3.5

Chainarong Wongteerasupaya , Analsis of Genelic diversity in Ribonucleotide reductase gene of white spot sndrome virus **งานวิจัยอณูชีววิทยา ในหนังสือ** กลุ่ม เมธีวิจัยอาวุโส สกว. ศ. ดร. สกล พันธุ์ยิ้ม 19 กันยายน 2545 หน้า 9/1-6

ANALYSIS OF GENETIC DIVERSITY IN RIBONUCLEOTIDE REDUCTASE GENE OF WHITE SPOT SYNDROME VIRUS

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Abstract

The genetic variations among WSSV isolates were study by comparing the direct repeat DNA sequence of coding in ribonucleotide reductase genes. We have found high variation in the number of 54 bp DNA repeats in the WSSV ribonuclease reductase gene. The sequence within the repeated region was highly stable showing sequence variation for some repeats at position 36 of the 54 bp repeat only and even then for an alternate T or G only. This method of comparison may be useful for epidemiological studies on the local to global movement of WSSV since it shows high variation in the number.This direct repeat sequence probably be the candidated gene to distinquish between the WSSV isolates of penaeid shrimp. The sequence of difference PCR repeating patterns are understudies.

Introduction

White spot syndrome virus (WSSV) is a major viral disease agent inshrimp culture. Electronmicroscopy studies showed that the virions are enveloped and have a bacilliform shaped of 275 nm in length and 120 nm in width with a tail-like appendage of one end (Wongteerasupaya et al.,1995). Nucleocapsides, which have lost their envelope, has a cross-hatched appearance and a size of 300 x 70 nm (Wongteerasupaya et al.,1995). This viral morphology, its nuclear localization, and its morphogenesis are'reminiscent of baculoviruses in insects (Durand et al.,1997). The phylogenetic analysis of ribonucleotide reductase gene indicated that WSSV belongs to the eukaryotic branch of an unrooted parsimonious tree and suggest to be a number of a new genus (Whispovirus) within the baculoviridae or a number of an entirely new virus family (Van Hulten et al.,1999). Other genes that are highly conserved in related virus are those coding for viral capsid and envelope protein (Morphy et al.,1995). This two WSSV virion protein showed no homology to known baculovirus protein in databases.

The 12.3 kb ribonucleotide reductase gene was reported by van Hulton et al., 2000. This segment contained eight open reading frames(ORFs), including two encoding the large (RR1) and small (RR2) subunits. The rr1 andrr2 genes were separated by 5760 bp, containing several putative ORFs and two domains with multiple sequence repeats. The first domain contained six direct repeats of 54 bp and is part of a coding region. The second domain had one partial and two complete direct repeats of 235 bp at an intergenic region. The phylogenetic analysis of rr1 and rr2 indicated that WSSV belongs to the eukaryotic branch of an unrooted parsimonious tree.

The virus has a wide host range among crustaceans (Flegel,1997) and distinctive clinical signs inpenaeid shrimp. There is little genetic variation among WSSV isolates from around the world using combinning the method of restriction analysis and southern blot hybridization. In this paper, we study the relationships between WSSV isolates based on sequence analysis of viral enveloped protein VP28 gene, and the segment of six direct repeats in coding regions of ribonucleotide reductase gene.

Material and Method

The virions were isolated from infected *penaeus monodon* shrimp from Thailand.Infected tissue was homogenized in TN buffer (20mM Tris-HCl,400 mM NaCl,pH 7.4) after centrifugation at 1700 g for 10 min,the viral DNA was isolated by treament with proteinase K (0.2mg/ml) and sarkosyl(1%) at 45°C for 2-4 hr. followed by phenol-chloroform extraction.

Primer and PCR reaction

PCR primer of segment of six direct repeats of ribonucleotide reductase gene

Wrb6r-F AGCAGGTGTGTACACATTTCATG

Wrb6r-R TCTACTCGAGGAGGTGACGAC

The PCR reaction,10 ng DNA and 30 pmol each primer were added to a reaction Mixture comprising 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2mM $MgCl_2$,200µM each dATP,dTTP,dCTP and dGTP, 25U Taq Pwo DNA polymerase (Gibco-BRL). The mixture was incubated in a thermal cycler using 30 cycle of 94°C for 30s, 52°C for 30s,and 72°C for 1 min and a final elongation step of 72°C for 10 min. PCR products were purified using a QIA quick™ column(QIAGEN).

Sequence analysis

PCR amplicons were purified from agarose gels using a QIA quick Gel. Extraction Kit (QIAGEN) and were sequenced using thermo Sequence™ (Amersham) and an Automated ABI model 377 Sequencing system (Applied biosystem Inc.). Sequence alignment and estimation of sequence identity were conducted using Clustal W software.

Results and discussion

The DNA extracts from WSSV infected shrimp gave a variety of amplicons using the primers Wrb6r-F and Wrb6r-R , suggesting differences in the number of repeats from sample to sample. An example gel for fragments representing 6 to 11 repeats is shown in Fig. 1. In GenBank records of for complete WSSV genome sequences, one originating from China shows 12 repeats (AF332093) and two show 6 repeats, one from Thailand (AF369029) and one from China (AF440570). This supports our results regarding variability in the number of RR54 repeats for WSSV.

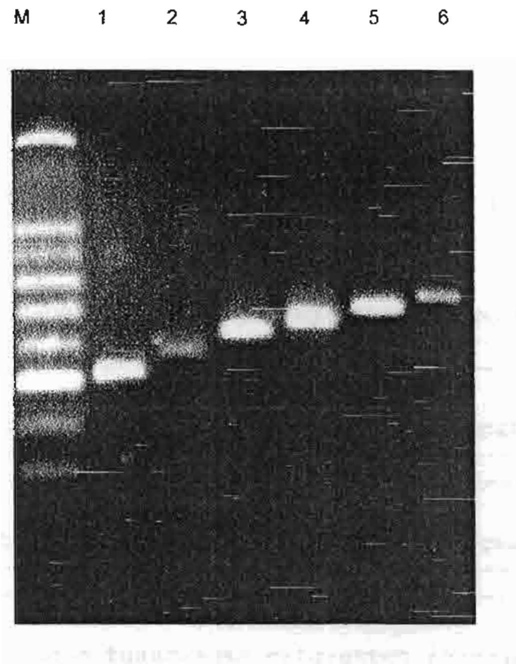

Figure 1. Example agarose gel showing PCR amplification products for 6 (lane 1) to 11 (lane 6) RR54 repeats.

Sequencing of several of the fragments confirmed that they comprised variable stretches of tandemly repeated RR54 with almost unvarying homology to RR54 in the original WSSV sequences deposited at GenBank (Figs. 2). Sequence variations, when found, consisted of a single alternate T or G at position 36 of the 54 bp repeat. This was also characteristic of the GenBank sequences. In the initial repeat (RR54-1), T at position 36 was invariant in all the sequenced fragments and for the full WSSV sequences at GenBank. T was also invariant for RR54-2 except for G found in GenBank AF369029 originating from Thailand (Table 1).

```
Ref 1: 142667 tc tactcgagga ggtgacgacg acgacgacga tgac
Ref 2: 127310 ------------ ---------- ---------- ----
Chum4:        ------------ ---------- ---------- ----

Ref 1: gatggaggaa ctttcgatac agtagggtct ggtatacttg gacgcaaaaa gcgt
Ref 2: ---------- ---------- ---------- ---------- ---------- ----
Chum4: ---------- ---------- ---------- ---------- ---------- ----

Ref 1-RR54-1: gccgcacctc cacctgagga tgaagaagag gatgatttct accgcaaaaa gcgt
Ref 2-RR54-1: ---------- ---------- ---------- ---------- ---------- ----
Chum4-RR54-1: ---------- ---------- ---------- ---------- ---------- ----

Ref-RR54-2:   gccgcacctc cacctgagga tgaagaagag gatgagttct accgcaaaaa gcgt
Ref 2-RR54-2: ---------- ---------- ---------- -----t---- ---------- ----
Chum4-RR54-2: ---------- ---------- ---------- -----t---- ---------- ----

Ref-RR54-3:   gccgcacctc cacctgagga tgaagaagag gatgagttct accgcaaaaa gcgt
Ref 2-RR54-3: ---------- ---------- ---------- -----t---- ---------- ----
Chum4-RR54-3: ---------- ---------- ---------- -----t---- ---------- ----

Ref-RR54-4:   gccgcacctc cacctgagga tgaagaagag gatgagttct accgcaaaaa gcgt
Ref 2-RR54-4: ---------- ---------- ---------- ---------- ---------- ----
Chum4-RR54-4: ---------- ---------- ---------- ---------- ---------- ----

Ref-RR54-5:   gccgcacctc cacctgagga tgaagaagag gatgatttct accgcaaaaa gcgt
Ref 2-RR54-5: ---------- ---------- ---------- ---------- ---------- ----
Chum4-RR54-5: ---------- ---------- ---------- -----g---- ---------- -- -

Ref-RR54-6:   gccgcacctc cacctgagga tgaagaagag gatgatttct accgcaaaaa gcgt
Ref 2-RR54-6: ---------- ---------- ---------- ---------- ---------- ----
Chum4-RR54-6: ---------- ---------- ---------- -----g---- ---------- ----

Ref 1: taaactacgc acgaaagtga cggtggttga aaatagacta atattgttga tatg
Ref 2: ---------- ---------- ---------- ---------- ---------- ----
Chum4: ---------- ---------- ---------- ---------- ---------- ----

Ref 1: ttaacccctt tttttcatga aatgtgtaca cacctgct 143172
Ref 2: ---------- ---------- ---------- -------- 127816
Chum4: ---------- ------
```

Figure 2. Example of sequence for the amplicon for Chum4 showing identical repeat sequences to the reference strain (GenBank AF369029 = Ref 1 and AF440570 = Ref 2) except for the bases indicated in bold at position 36 of RR54. Matching bases are indicated with a dash and the primers are underlined.

Table 1. Summary of substitutions found at position 36 in sequenced RR54 tandem repeat regions of selected amplicons from Thai shrimp samples from Surat Thani (Surat) and Chumpol (Chum) compared to GenBank references with 6 and 12 RR54 repeats.

Isolate	RR54 repeat number											
	1	2	3	4	5	6	7	8	9	10	11	12
Surat #1	T	T	T	T	G	T	T	G	T			
AF440570	T	T	T	G	T	T						
Chum #2	T	T	T	G	T	G	T					
Chum #4	T	T	T	G	G	G						
Surat #2	T	T	G	T	T	G	G	T				
Chum #3	T	T	G	T	T	G	G	T				
AF332093	T	T	G	G	G	G	G	G	T	T	T	T
AF369029	T	G	G	G	T	T						

Obviously, the number of samples employed in this study was too small and taken at too intermittent intervals to make firm conclusions. However, they do show that the RR54 repeat region has potential for use in epidemiological work with WSSV and may help in tracing its origin and spread.

Acknowledgements.

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5.3.6

Richard Hodgson and **Chainarong Wongteerasapaya** , Nucleic Acid Hybridization to detect YHV and WSSV Molecular Epidemiology and Diagnostics of Shrimp Viruses in the Asian Region . March 2000. P5/1-12

NUCLEIC ACID HYBRIDIZATION TO DETECT YHV AND WSSV

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SECTION 1 INTRODUCTION

Preface

Dot blots, Southerns and Northerns involve the adsorption and permanent binding of nucleic acid onto a membrane support prior to an hybridization assay. In dot blots an unfractionated nucleic acid sample is permanently cross-linked to the membrane. In the Southern and Northern blots fractionated DNA and RNA respectively are transferred and cross-linked to a membrane after fractionation through a gel.

A nucleic acid sample that is permanently immobilised onto a membrane support can be probed for the presence of specific nucleic acid target sequence. Probes are made to complement the sequence of the target. In this way the probe will bind (hybridize) to the target. Probes are labeled with a reporter molecule, such as DIG, biotin, ^{32}P or a fluorophore for detection of the hybridization.

Blotting

Dot blots use small sample volumes as low as 1µl to test whether the sample contains the target sequence of interest. Crude or purified samples can be examined and the system is applicable for detection of DNA and RNA targets. Southern blots examine DNA, while Northern blots examine RNAs.

One of the main advantages of the dot blot method is the ability to examine a large number of samples in a short time and on a small membrane area. This is an ideal method for initial screening of sample and for diagnostic screening of samples when the specificity of the probe for the target has been established.

The dot blot assay will only provide presence/absence information about a sequence. No information on the size or structure of the target in a positive sample can be obtained. This additional information is gained after blotting nucleic acid fractionated by gel electrophoresis.

DNA is transferred after alkaline denaturation or in an alkaline solution to disrupt the H+ bonds which hold strands together in the DNA duplex. It is necessary to dissociate DNA strands to allow the probe to hybridize to one of the strands. The alkaline conditions used for DNA transfers cause random cleavage in RNAs. So RNA is generally transferred using a high salt solution buffered to neutrality and containing a denaturing agent. The bound single stranded RNA can then be detected.

The type of membrane used as the support is important. There are three groups of membranes; nitrocellulose, uncharged nylon and (+ve) charged nylon. For each different membrane there is a slightly different procedure required for preparing the samples and the membrane.

Hybridization of probe to samples in a gel is generally not efficient. So nucleic acid is transferred to a membrane support for efficient hybridization. The profile of the fractionated nucleic acid bands in the gel is maintained during the transfer to membrane.

Transfer and binding of RNA onto membranes from gels is termed Northern transfer. The equivalent for DNA is a Southern transfer.

Hybridization

Molecular hybridization of a probe to a target nucleic acid species is a powerful technique to 'find' whether the target species is in a sample. Hybridization is the basis of nearly every procedure in molecular biology. So understanding how hybridization conditions work provides an understanding of the probe-target interaction.

In an hybridization assay the target can be RNA or DNA and the probe is a nucleic acid. The hybridization can occur on fixed membrane supports, on glass or magnetic beads, in microtitre plates, or in solution or in a gel matrix. In most circumstances the probe is labelled with a reporter group for detection following hybridization. Many different reporters are available, each with their advantages. The choice of reporter depends on each laboratory situation.

The efficiency of hybridization is controlled through the interaction of many variables such as, probe base composition (G+C% plus A+T%), mismatch percentage, concentration, size of the probe and whether the hybridization involves RNA/RNA, DNA/RNA or DNA/DNA duplexes. Also of importance are the ionic concentration, solution viscosity and presence of destabilising agents in the reaction solutions, and the hybridization and washing temperatures. All these features combine to vary the stringency of the hybridization and can be related together for comparison between probes through the Tm (melting temperature) for hybridization of the probe/target.

Stringency is the specificity and strength of probe-target hybridization. High stringency allows only complete base pairing but at low stringency a level of base pair mismatch is tolerated. Tm is the temperature where 50% of the probe is expected to be hybridized to target.

Large probes made but random priming, by PCR, by nick translation or by transcription typically produce probes of 100 to 500 nt and they can be DNA or RNA. Because of their size Large probes have very high Tm, which gererally requires formamide in the hybridization to lower the effective Tm to a manageable temperature. Full-length probes require an extended hybridization period and they can tolerate considerable base-mismatch without markedly affecting the hybridization outcome. It is difficult for full length probes to distinguish closely related individuals or groups. They are therefore best used as a general probe.

Oligonucleotide (oligo) probes are small (25 to 45 nt) and they have a sequence defined by the user. Thus, they hybridize to a defined target sequence, which can be a motif unique to a pathogen species. If this sequence motif is unique and the oligo-probe is used under high stringency conditions then the probe is specific for its target pathogen species or strain. These are the *diagnostic probes.* In comparison, if the target sequence is found among a family of pathogens, then an oligo-probe can be developed which universally detects any member of the family, but does do detect unrelated families. These are the *general probes.* Oligo-probes rapidly diffuse in solution, so they allow for rapid hybridization conditions. They are easily synthesised in large quantity, they are a stable and very economical.

Often when total nucleic acids are fractionated through an agarose gel they often show a staining smear which may or may not contain visible fragment bands. Nucleic acid (bands) of interest are difficult to visualise because of the high total nucleic acid concentration or because the bands of interest occur at a low concentration below the detection by staining. To overcome this problem the particular target sequences are detected by hybridizing a probe which is labelled with a detectable marker. Markers can be a radiolable (^{32}P, ^{35}S), biotin, digoxigenin, a fluorophor or even directly coupled enzymes. With each marker the sensitivity of detection is much greater than gel staining. However, hybridization of probe to samples in a gel is generally not efficient. So nucleic acid is transferred to a membrane support for efficient hybridization. The profile of the fractionated nucleic acid bands in the gel is maintained during the transfer to membrane.

Transfer and binding of RNA onto membranes from gels is termed Northern transfer. The equivalent for DNA is a Southern transfer.

Hybridization probes can usually be designed to detect a specific target sequence. Alternatively there are the broad spectrum probes, which are used to detect different but related targets. That is they share a region of homology. This is the difference between diagnostic and general probes. Thus, the selection of the probe and the assay conditions must be carefully considered and controlled before performing a dot blot hybridization.

Reporter molecules attached to probes

The reporter molecules, or labels, are either radioactive or non-radioactive. Radioactive labels are widely used and have many advantages. They are not dangerous if used correctly, which is how all laboratory chemicals need to be used. The labeling and detection systems outlined below can be done using commercially available kits.

The non-radioactive labels can be fluorescent groups, which are detected directly in specialised equipment, or the labels can be reactive chemical groups. The two most common chemical labels are biotin and DIG (digoxigenin). These groups are detected after the hybridization in an additional sandwich assay involving attachment of an enzyme which catalyses the final detection signal. Biotin and DIG detection involving colourmetric development is relatively simple but it is less sensitive than a chemiluminescent detection system.

Reporter Molecules For Labelling Nucleic Acid

Radioactive
- ^{32}P, ^{33}P and ^{35}S

Non-radioactive
- DIG (digoxigenin)
- biotin
- fluorophores (many available)
- conjugated enzymes

Radiolabels

The commonly use radioactive labels contain 32phosphorous (^{32}P) or ^{33}P, or to a much lesser extent now ^{35}S.

^{32}P has very high energy emission which provides a fast exposure times to film and screens. But a limitation is that developing bands can lose resolution. Generally this is not a problem. As an alternative, ^{33}P can be used in all the same applications. It has less energy emission so development times have to be increased, but resolution is improved. The lower energy emission is also safer for handling.

Each of the deoxyribo-nucleotides and the ribo-nucleotides can have ^{32}P incorporated into their structure. However, the most common labels are (dATP, dCTP, ATP and UTP).

DIG (Digoxigenin)

DIG is a highly antigenic steroid from Foxglove plants (*Digitalis purpurea*). As a label DIG is attached to a spacer arm which is attached to a uridine residue. The uridine is enzymatically incorporated into the probe. DIG can also be chemically attached to molecules. The chemistry of DIG together with labelling procedures and uses of DIG-probes are extensively reviewed in Boehringer Mannheim User's Guides. DIG labelling is generally easy and the probes are stable for a long period. It is also possible to purchase oligonucleotide probes with the DIG already attached. An advantage with DIG is that it is only reported to occur in the Foxgloves. So unless extracts from these plants are being examined there is little problem with background contamination due extraneous label.

Biotin

Biotin can be incorporated into probes in the same manner as DIG. Biotin can also be incorporated by direct cross-linking under UV-light. Some plants have an intrinsic level of biotin which can lead to high background.

Flurophores

Fluorophore	Emission
Cascade Blue	blue-420
AMCA	blue-448
Oregon Green 488	green-514
Fluorescein	green-520
FAM	green-535
Bodipy	green-533
JOE	yellow-557
HEX	yellow-560
Cy 3	orange-570
Tetramethylrhodamine	orange-580
TAMRA	orange-580
Rhodamine Red, ROX	red-590
Texas Red	red-615
Cy 5	far-red-670

These are molecules which absorb light, but are metastable and re-emit light at a longer wayelength as they regain there stable state. Application of flurophores is becoming increasingly common. They offer particular advantages because multiple flurophores can be examined simultaneously in a sample. This means several probes can be used simultaneously in the one reaction to detect different targets.

Conjugated enzymes

The enzymes alkaline phosphatase (AP) or horseradish peroxidase (HRP) can be directly conjugated onto probes or primers. Although this is not common. They are extensively used in protein probes (antibodies) where they can be easily attached.

Detection Of Reporter Molecules

Reporter molecules are detected either directly or indirectly through a secondary reaction, which often amplifies the detection. Radiolabels, fluorophores and the coupled enzymes are detected directly. DIG and Biotin are detected indirectly by binding an antibody-enzyme conjugate. The enzyme catalyses a colourimetric or luminescent reaction which is recorded.

The radio-labels are detected by exposure to X-ray film or to fine grain photographic film followed by photographic development of the films. More sophisticated detection involves exposure to specialised luminescent phosphor screens with development in a phosphorimager. Other specialised equipment such as liquid scintillation counters can be used in some instances. Using ^{32}P and X-ray film exposure is a reliable, sensitive and economical detection strategy for most application.

DIG provides a high level of sensitivity, which is achieved by an additional sandwich assay involving an anti-DIG antibody coupled with an enzyme which catalyses the final detection signal. The enzyme induced signal can provide a colourmetric development from BCIP/NBT or the enzyme induced signal can stimulate a chemiluminescent detection which is very sensitive and rapid.

The colourmetric development or chemiluminescent detection as described for DIG is also applicable for biotin. Flurophores are detected directly in plate readers, in flat bed readers which examine plates, membranes and gels, or they can be detected by microscopy under UV-light excitation.

The directly coupled enzymes are not a common labelling technique. They are detected by colour development or chemiluminescence.

Molecular detection techniques for viruses are based on the identification of a specific molecular component(s) of the virus of interest. This could be virus specific protein or nucleic acid. The sensitive methods for detection of virus specific proteins are achieved using immunological techniques. For example by ELISA (enzyme-linked immunosorbent assay), DIBA (dot immunobinding assay) and immunofluorescence. Proteins are highly immunogenic which makes them very suitable to immuno-based detection procedures.

The genetic material of all life forms (nucleic acid) is a poor antigen, so the direct immuno-based methods used for protein detection of viral specific nucleic acid are not appropriate for the detection of nucleic acid. Traditional procedures used for detection of nucleic acid, e.g. those involving nucleic acid hybridization and PCR can however be converted into an immuno-based detection procedure by incorporating an antigen into the nucleic acid probe.

Nucleic acid probes can be labelled with digoxigenin (DIG), biotin or enzymes directly. These are all antigenic and allow the labelled probes to be inicorporated into an immuno-based detection strategy.

DIG is a commonly used label for incorporation into nucleic acid. Its detection is by and anti-DIG antibody coupled to a reactive enzyme, such as alkaline phosphatase. The phosphatase can be use to react with different substrates. When chromogenic substances like BCIP/NBT are used a colour precipitate develops, which is the final product detected to indicate the presence of a positive reaction.

SECTION 2 REAGENTS AND MATERIALS

Sample preparation

- 2X, 6X and 10X SSC: diluted from stock 20X SSC solution (3 M NaCl, 0.3 M sodium citrate, pH 7)
- 0.4 M Tris-HCl pH 7.5: diluted from stock 10 N solution
- 0.25 N HCl: diluted from stock 10 N solution
- 0.2 N and 0.4 N NaOH: diluted from stock 10 N solution
- Formamide: deionised 95% formamide
- Formaldehyde: stock solution is usually 37% (≡ 12.3 M)
- 5X MOPS buffer: 0.1 M MOPS (3-(N-morpholino) propane-sulphonic acid) pH 7.0, 40 mM sodium acetate, 5 mM EDTA
- DSW: DEPC-treated Sterile double distilled Water

Hybridization with random-primed probes

- PHB: 5X SSC, 1% (w/v) blocking reagent, 0.1% (w/v) N-lauroyl sarcosine, Na-salt, 0.02% (w/v) SDS
- Membrane wash solution 1: 2X SSC, 0.1% (w/v) SDS
- Membrane wash solution 2: 0.1X SSC, 0.1% (w/v) SDS
- Buffer I: 10 mM Tris-HCl, 150 mM NaCl, pH 7.5
- Buffer II: 1% (w/v) blocking reagent in buffer 1
- Buffer III: 100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5
- Colour solution: 45 □l NBT solution, 35 □l X-phosphate solution in Buffer III
- NBT: 75 mg/ml nitro blue tetrazolium in 70% (v/v) dimethylformamide
- X-Phosphate: 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt in 100% dimethyforamide

Hybridization with oligonucleotide-probes

- 20X SSC buffer: 3M NaCl, 0.3M trisodium citrate
- Prewash solution: 0.1X SSC, 0.5% SDS
- Prehybridization solution: 5X SSC, 20mM NaH_2PO_4-pH7.0, 2.5% SDS, 5X modified Denhardts, 100μg/ml denatured DNA (RNAse free)
- 100X Modified Denhardts solution: 2% polyvinylpyrrolidone 40, 2% Ficoll 400, 2% polyethylene glycol 8000
- Denatured DNA: 10mg/ml herring or salmon sperm DNA, 0.1M NaCl. Sheared and free of protein. (NOTE, substitution with 10mg/ml yeast tRNA is acceptable for Northerns)
- Wash solution one (1): 3X SSC, 2.5% SDS
- Wash solution two (2): 0.5X SSC, 2.5% SDS
- Wash solution three (3): 0.2X SSC, 1% SDS
- Membrane stripping solution: 1mM Tris-HCL pH 8.0, 1mM EDTA, 0.2X modified Denhardts

Colour development to detect DIG and Biotin probes

- Phosphate buffered saline (PBS): 8g/l NaCl, 0.2g/l KH_2PO_4, 1.15g/l Na_2HPO_4, 0.2g/l KCl pH 7.4
- Blocking buffer A: to PBS add skim milk powder to a concentration of 2.6% w/v
- Blocking buffer B: to 9 ml of blocking buffer A add 1ml of slurry from a healthy *animal.* Centrifuge at 3000rpm x 5min to sediment debris
- Washing buffer AP 7.5: 0.1M Tris-HCl pH 7.5, 0.1M NaCl, 2mM $MgCl_2$, 0.05% v/v Triton X100
- Washing buffer AP 9.5: 0.1M Tris-HCl pH 9.5, 0.1M NaCl, 5mM $MgCl_2$
- Stop buffer: 10mM Tris-HCl pH 7.5, 5mM EDTA
- Antiserum to reporter: diluted to 1/250 in blocking buffer B
- Alkaline phosphatase conjugated goat anti-rabbit gamma-globulin: diluted to appropriate working concentration (1/1000) in PBS + 1% w/v bovine serum albumin (BSA)
- Phosphatase substrate stock solutions: Immediately before use add 44μl of NBT and 33μl of BCIP to 10ml of AP 9.5.
- NBT = Nitroblue tetrazolium (75mg/ml in 70% dimethylformamide)
- BCIP= Bromo-4-chloro-3-indolyl phosphate (50mg/ml in 100% dimethylformamide)

SECTION 3 PROCEDURES NOTES

Dot blot membrane templates

Use positively charged nylon membranes, nitrocellulose is not recommended

2 ALWAYS WEAR GLOVES WHEN HANDLING MEMBRANES. It is essential to keep the membranes clean and free of finger moisture and oils, which inhibit nucleic acid binding

3 For large sample numbers a template based on a 100 dot design is used

4 A 100 dot blot template is given below

5 Copy the template below onto the membrane with a pen (e.g.. 045 Reynolds Fine Carbure, made in France), soft pencil or stamp which will not fade out during the hybridization washes. It is advisable to mark out duplicate membranes to prepare replicate membranes

6 For each membrane cut out 2 pieces of absorbent paper, which are slightly larger than the membrane (to cover the membrane) and a piece of clean filter paper

7 Membrane template for 100 place Dot blot. The first column (-1) is used for a dilution series of the positive standard and for negative controls.

0	1	2	3	4	5
A					

	0	1	2	3	4	5	6	7	8	9	1
	A										
	B										
	C										
	D										
	E										
	F										
	G										
	H										
	I										
	J										

Map and description of samples on dot blot membrane

-1	0	1	2	3	4	5	6	7	8	9	10
	A										
	B										
	C										
	D										
	E										
	F										
	G										
	H										
	I										
	J										

Preparation of the sample
Loading membranes

- Isolate a shrimp total nucleic acid (TNA) fraction by a standard procedure or obtain a nucleic acid fraction from purified virus

DNA Dot Blot

1. Wet the membrane in 0.4 M Tris-HCl, pH 7.5 and soak for 5 min
2. Denature DNA for 5 min at 95°C
3. Immediately transfer the DNA to ice and quick chill
4. Spot 2 to 5 µl of sample onto positive nylon membrane and allowed to air dry
5. Denature the DNA using 0.2 N NaOH for 5 min
6. Cross-linked the samples to the membrane by exposure to UV light (254 nm, 125 mJ total) or by fixation at 80°C x 2h under vacuum. Store membranes dry
7. Soak the membrane in 6X SSC for 1 min. before hybridization

RNA Dot Blot

1. Wet the membrane in SW for 15 min.
2. In a microfuge tube prepare the sample as follows:

Deionized formamide	17.5 µl
37% (w/v) formaldehyde	6.0 µl
5X MOPS, pH 7.0	3.5 µl
RNA	variable
DSW to a final volume of	35 µl

3. Spot 2 to 5 µl of sample onto positive nylon membrane and allowed to air dry
8. Cross-linked the samples to the membrane by exposure to UV light (254nm, 125mJ total) or by fixation at 80°C x 2 h. under vacuum. Store membranes dry
4. Soak in 6X SSC for 1 min. before hybridization

Southern and Northern blot transfer

Preparing the gel

- Note: Wear gloves during this procedure to stop ethidium bromide getting on fingers and to stop grease transferring from hands onto membranes.

1. The following is based on mini gels (6.5 x 10cm) but is directly applicable to larger gel formats
2. Remove the prestained gel from the gel tray and observe under UV light to ensure good gel fractionation has occurred and that no sample bands are more than 60mm from the origin.
3. Photograph the gel
4. Slice the gel just above the wells and discard this small top piece
5. Trim the gel to 70 mm from the new top just above the wells
6. Slice off the bottom right corner for gel orientation

The capillary transfer process

- Completely wet a sponge (size about 3cm thick by 10x15cm) with small pores in clean water

7. Squeeze out all water then soak the sponge in 20mM NaOH solution in a container so that the solution is just below the top of the sponge. Squeeze out all air bubbles

8. Take a piece of thin but firm plastic, which is slightly larger than the sponge, and neatly cut a hole 66 x 70mm for one gel (or 135 x 70mm for two gels) to make the gel template

9. Cut four clean pieces of Whatman filter paper, or equivalent, to 66 x 70 mm (or double size if transferring two gels)

10. Cut a piece of nylon membrane (Zeta Probe™) the same size, then along the bottom edge on one side write the gel number and date and trim off the bottom left corner. The non-written side will be the nucleic acid side after transfer

11. Cut some absorbent paper to a size marginally larger than the filter paper

Note : **It is essential not to have any air bubbles trapped between any layers in the capillary transfer setup** (except perhaps in the gel wells)

12. Carefully make the gel sandwich for nucleic acid transfer

13. Place a dry plastic gel template in the centre on the sponge, be sure not to wet the top of the template

14. Wet a piece of filter paper in the transfer solution and lay it in the hole of the plastic gel template

15. Repeat with another piece of wet filter paper

16. Place the gel on the filter paper so the cut corner is now on the bottom left side (i.e. the original upper surface of the gel faces down and is in contact with the filter papers)

17. With a pipette gently apply transfer solution under the gel to remove all air bubbles

18. DO NOT PROCEED UNTIL ALL AIR BUBBLES ARE REMOVED

19. Gently lower the membrane into the transfer solution until the membrane is completely wet

20. Lay the wet membrane over the gel so cut corners align and you can read the writing

21. Wet one piece of filter paper and lay over the membrane

22. Repeat with another piece of filter paper

23. Carefully place the absorbent paper on the filter paper to a height of about 8cm

24. Finally place a weight on the absorbent paper to facilitate good contact for capillary movement, but do not unduly squash the sandwich

25. Proceed with the transfer for 4 - 16h when examining low molecular weight samples, or transfer overnight (~16-20h) if examining high molecular weight samples

26. After the transfer period remove all wet papers, remove the membrane and place it on dry paper so the side with writing faces down and the nucleic acid side therefore faces up

27. Covalently attach the nucleic acid to the membrane by exposure to 125mJ of UV light (254nm) or by baking at 80°C for 2h