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Detection of yellow-head virus (YHV) of *Penaeus monodon* by RT-PCR amplification

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ABSTRACT: A nucleic acid probe was developed using cDNA prepared from ssRNA extracted from yellow-head virus (YHV), a serious pathogen of the black tiger prawn *Penaeus monodon*. The specificity and sensitivity of this probe was established using dot-blot hybridization with nucleic acid extracts from YHV and from shrimp, bacteria and other viruses. Based on the sequence of this cloned YHV cDNA fragment, a YHV specific primer set for reverse transcription polymerase chain reaction (RT-PCR) of a 135 base pair (bp) sub-fragment was designed for detection of YHV infections in penaeid shrimp. When applied in RT-PCR with templates derived from experimentally or naturally YHV-infected shrimp and with purified YHV or YHV nucleic acid, the expected 135 bp amplification product was obtained. By contrast, nucleic acids extracted from tissue samples of healthy shrimp and from other shrimp pathogens gave no such fragment. This confirmed the specificity of the designed YHV RNA specific primers. RT-PCR based detection demonstrated high sensitivity, in that it could detect 0.01 pg of purified YHV-RNA. In a time course study of an experimental YHV infection, the RT-PCR detection showed evidence of infection at 6 to 12 h post exposure to the virus. However, histopathology typical of YHV infection [i.e. karyorhexis and pycnosis of haemocytes in haematoxylin and eosin (H&E) stained haemolymph smears] was not visible until 42 to 48 h post exposure. The results suggested that RT-PCR might be useful to shrimp aquaculturists for early detection of YHV outbreaks or for detection of asymptomatic carriers.

KEY WORDS: Yellow-head virus (YHV) · Polymerase chain reaction (PCR) *Penaeus monodon*

INTRODUCTION

Yellow-head virus (YHV) of the black tiger prawn *Penaeus monodon* was first discovered in Thailand in 1992, although it is now known to have caused extensive losses on the eastern coast of the Gulf of Thailand as early as 1991 (Flegel et al. 1995). Shrimp infected with YHV often show light yellow coloration of the dorsal cephalothorax area and have a pale or bleached appearance (Limsuwan 1991). Upon initial discovery, the virus was considered to be a granulosis-like virus (Boonyaratpalin et al. 1993, Chantanachookhin et al. 1993), but later work showed that it was actually an RNA virus (Wongteerasupaya et al. 1995a). Two viruses which morphologically resemble YHV have also been reported in lymphoid organs (Spann et al. 1995) and gills (Spann et al. 1998) of *Penaeus monodon* from Australia. The virus from Thailand is also known to infect other species of penaeid shrimp in laboratory tests (Lu et al. 1994, 1997, Lightner 1996, Flegel et al. 1997), so it is not a potential pathogen solely to *P. monodon*. Since YHV is an RNA virus, cDNA preparation was necessary in order to characterize its nucleic acid and to prepare a diagnostic probe. The final aim was to develop a rapid, simple and sensitive reverse transcription polymerase chain reaction (RT-PCR) based system that would allow early detection of the virus.

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MATERIALS AND METHODS

Viral isolation and nucleic acid extraction. YHV was isolated from experimentally infected juvenile shrimp *Penaeus monodon* as described by Wongteerasupaya et al. (1995b). The YHV RNA was extracted from purified virions using guanidinium thiocyanate and was purified by CsCl gradient ultracentrifugation as described by Wongteerasupaya et al. (1995a).

RNA extraction by Trizol™ reagent. To prepare total RNA extracts for use as RT-PCR templates, 50 mg of shrimp gill tissue or 50 µl of haemolymph was treated with Trizol™ reagent for extraction of RNA (Bethesda Research Laboratories, Gaithersberg, MD, USA), following the instructions in the reagent manual.

Preparation of double-stranded cDNA. Using RNA extracted from purified YHV as a template, cDNA was prepared at 37°C for 50 min, in 20 µl of reaction mixture containing 2.5 µM random hexamer (Sigma), 50 U Maloney murine leukemia virus reverse transcriptase (M-MLV) (Bethesda Research Laboratories, Gaithersberg, MD), 20 U of RNase inhibitor (Bethesda Research Laboratories, Gaithersberg, MD), 1 mM each deoxyribonucleotide triphosphate (dNTP), 5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3). Using 2 to 5 ml of this, double-stranded cDNA was synthesized in a final volume of 50 µl reaction mixture containing a final concentration of 2 mM $MgCl_2$, 0.1 mM each dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.3) and 2.5 µM random hexamer. The sample was subjected to amplification in a DNA thermal cycler (Perkin Elmer Cetus) at 95°C, 30 s; 20°C, 5 min; and 37°C, 2 min, for 8 cycles with the addition of 1 µl of 5 U μl^{-1} of Klenow fragment enzyme (Boehringer Mannheim Genius System) at each annealing step.

Cloning and screening of cDNA clones. The cDNA was digested with Sau3AI and the resulting fragments were ligated to *Bam*HI digested Bluescribe plasmid (Stratagene) and transformed into *Escherichia coli* JM107. Transformants were selected on Luria-Bertani-agar containing 50 µg ml^{-1} ampicillin, 1 mM Isopropyl-D-thiogalacto-pyranoside (Sigma) and 2% 5-bromo-4-chloro-3-indoyl-galactoside (Sigma). Selected cDNA clones were then used as probes in dot-blot hybridizations with YHV RNA and nucleic acid preparations from various other sources. These included 1000, 100, and 10 ng of YHV RNA, shrimp genomic DNA, white-spot baculovirus DNA (WSBV) and control transcribed RNA from plasmid PAW109 (Perkin Elmer Cetus). Clones which gave positive hybridization only with YHV RNA were selected for further study.

DNA sequence and primer designation. The sequence of 1 selected YHV clone which gave good specificity for YHV-RNA was determined by the dideoxy chain termination method using Sequence kit version 2.0 (USB) and (α-35S) dATP (Amersham) with M13/mp18 oligonucleotides as the sequencing primer. Then nucleotide primers were designed by using the programme Oligo version 4.0.

Amplification of a YHV-specific fragment by reverse transcription polymerase chain reaction. Extracted YHV RNA was incubated at 42°C for 15 min to synthesize cDNA in 20 µl of PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl) containing 2.5 U of M-MLV reverse transcriptase, 1.0 U of ribonuclease inhibitor, 0.75 mM of antisense primer (144R), 1 mM each of dATP, dTTP, dCTP, and dGTP, and 5 mM of $MgCl_2$. Following cDNA synthesis, the mixture was incubated at 100°C for 5 min to inactivate the reverse transcriptase and then the product was allowed to cool to 5°C. The PCR mixture (10 mM Tris-HCl, pH 8.3, 50 mM KCl) containing 2.5 U of Taq DNA polymerase (Perkin Elmer Cetus), 2 mM $MgCl_2$ and 0.75 mM of sense primer (10F) was added to the reverse transcription product, giving a final volume of 100 µl. The tubes were overlaid with 100 µl of mineral oil. PCR amplification was carried out for 40 cycles at 94°C, 30 s; 58°C, 30 s; and 72°C, 30 s and finishing at 72°C for 10 min. In every set of experiments, a negative control was included. This contained diethylpyrocarbonate (DEPC)-treated distilled water instead of RNA. Amplified products were detected by electrophoresis of 20 µl aliquots through 2% agarose gels in Tris-Borate-EDTA (TBE) buffer.

Specificity of RT-PCR detection. Nucleic acid preparations including *Penaeus monodon* DNA, white-spot baculovirus WSBV DNA (Wongteerasupaya et al. 1995b), hepatopancreatic parvovirus (HPV) DNA (DiagXotics Co. Ltd, Wheaton, CT, USA), nuclear polyhedrosis virus (NPV) DNA (obtained from Rice Research Center, Kasetsart University, Thailand), *Salmonella* DNA and control plasmid PAW109 DNA (Perkin Elmer Cetus) were used as negative control templates for RT-PCR. From each RT-PCR reaction 20 µl was analysed by ethidium bromide stained gel electrophoresis.

Sensitivity of RT-PCR detection. Various amounts of YHV genomic RNA from 1 ng to 0.01 fg were used as templates in RT-PCR. Then 20 µl of each RT-PCR reaction mixture was analysed by ethidium bromide-stained gel electrophoresis.

Time course detection of YHV in experimental infection. Twenty-seven juvenile shrimp of 20 g average weight were infected with 0.1 ml of a thawed gill extract from experimentally YHV-infected shrimp (Wongteerasyupaya et al. 1995b). Fresh haemolymph (50 µl) was then collected from 3 of these infected shrimp every 6 h and immediately mixed with 500 µl of Trizol™ reagent to be subsequently extracted by the procedure described above. The resuspended RNA (2 µl) was then subjected to RT-PCR. Then 20 µl of each RT-PCR reaction was analysed by ethidium bromide-

stained gel electrophoresis. Haemolymph (0.1 ml) was also drawn from the sampled shrimp into an equal volume of 25% formalin and mixed thoroughly before making smears on microscope slides for staining with haematoxylin and eosin (H&E). Finally, the specimens were fixed in Davidson's fixative after haemolymph removal. Gills fragments from these fixed specimens were stained as whole fragments with H&E for microscopic examination as whole mounts (Flegel et al. 1995).

Southern blot hybridization. Southern blot hybridizations were carried out using digoxigenin-labeled DNA (Boehringer Mannheim Genius System) according to the Boehringer manual.

RESULTS

Dot-blot selection of pMUY412 probe

A total of 45 transformed, white clones were obtained on selective medium. Randomly, 12 clones were digoxigenin labelled and tested by dot blot hybridization. Clone pMUY412 [containing a 412 base pair (bp) insert of viral cDNA] was selected because it gave strongest positive hybridization with YHV genomic RNA but gave negative hybridization with healthy shrimp DNA, WSBV DNA and control transcribed RNA from plasmid PAW109.

Sequence analysis and primer design

The nucleotide sequence of the 412 bp cDNA fragment was analyzed and a set of PCR primers (10F of 21 bp and 144R of 20 bp) was designed to amplify a 135 bp fragment of YHV genomic RNA. The sequence of the 135 bp fragment (Fig. 1) gave no significant homology with the DNA sequence or putative protein sequences in existing international databases (i.e. Genbank, EMBL and SWISS PROTEIN databases).

Specificity of RT-PCR for YHV detection

In specificity tests of the YHV RT-PCR assay for YHV using 10F and 144R primers, 2 other economically important Thai shrimp pathogens (WSBV and HPV) and other nucleic acid templates (NPV, *Salmonella*, *Penadeus monodon* and plasmid PAW 109 DNA) gave no amplification product. The primers were shown to be specific for the selected region of the YHV genome (Fig. 2). A negative RT-PCR result was also obtained with 10 ng and 1 ng of purified YHV-RNA when the reverse transcription reaction step was omitted (Fig. 2; lanes 9 and 10).

CCG CTA ATT TCA AAA ACT ACG ACA GAA ACA
CCG GCA TGT CCT GTT CTC TCA CTG AAT TCC
AGC TCT CTC TCT CTC ACA TCC TCT ACC GTT
CTG AAG CAC AGC GTA CTC CTG ACG ACT TCC
TCG ACA TAA CAC CTT

Fig. 1. The sequence of the 135 bp YHV fragment in pMUY412. The underlined portion represents the position of the amplification primers 10F and 144R

Sensitivity of detection

To evaluate the sensitivity of the YHV RT-PCR assay, the reverse transcription and subsequent amplification reactions were carried out using purified viral genomic RNA. Various amounts of YHV-RNA were prepared by serial dilution in the range of 1 ng to 0.01 fg and subjected to RT-PCR for 40 cycles of amplification. When 20 µl of RT-PCR product from a 100 µl total reaction volume was directly analysed, the result showed that an amplification product could be visualized by ethidium bromide staining when as little as 0.01 pg of YHV-RNA was used as the template (Fig. 3A). Moreover, the sensitivity could be increased about 100 times when detection was by Southern blot hybridization using the digoxigenin-dUTP labeled pMUY412 parent fragment as a probe (Fig. 3B).

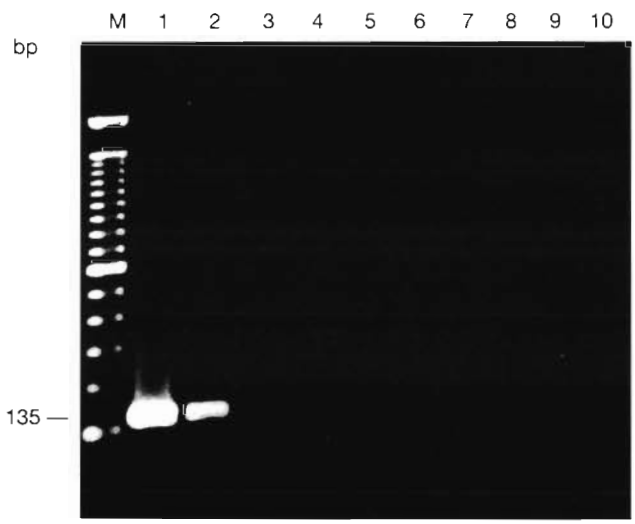

Fig. 2. Ethidium bromide staining of RT-PCR products amplified at an annealing temperature of 52°C for 40 cycles using 10 and 1 ng of YHV RNA (lanes 1 and 2), WSBV DNA (lane 3), HPV DNA (lane 4), NPV DNA (lane 5), *Salmonella* DNA (lane 6), healthy shrimp DNA (lane 7) and control template RNA from plasmid PAW109 (lane 8). No RT-PCR product was obtained for 10 ng and 1 ng of purified YHV RNA when the reaction omitted the step of reverse transcription (lanes 9 and 10). Lane M contained a 100 bp ladder marker

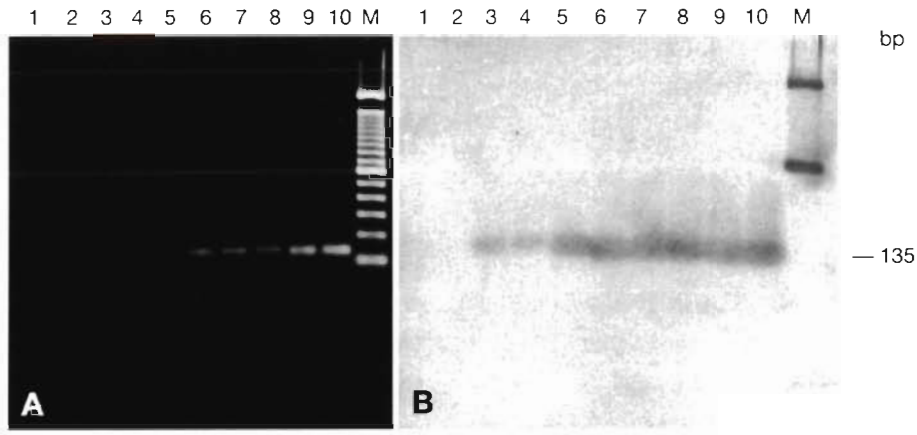

Fig. 3. Sensitivity of the RT-PCR assay. (A) Ethidium bromide staining of RT-PCR products amplified at an annealing temperature of 52°C for 40 cycles using serially diluted (1:10) YHV genomic RNA in the range of 1 ng to 0.01 fg as the template for RT-PCR (lanes 10 to 2, respectively). RT-PCR product at 20 µl from a total reaction volume of 100 µl was directly analysed. The gel in (A) was transferred by Southern blot for hybridization with Dig-dUTP labeled pMUY412 probe (B) No RT-PCR product was obtained using 10 ng of healthy shrimp DNA as the template (lane 1). Lane M contained a 100 bp size marker

Time course RT-PCR detection of YHV in laboratory shrimp infections

RT-PCR was carried out on haemolymph samples at various times after injection of YHV extracts into shrimp in the laboratory. At times 0, 6, 12, and 18 h post injection (p.i.), haemolymph was collected and RNA was extracted by Trizol™ reagent. The extract was subjected to RT-PCR using the optimized procedure described above. The results showed that RT-PCR could detect YHV very early during infection. It was found that 2 of 3 tested shrimp gave low to moderate RT-PCR products at 6 h p.i. At 12 and 18 h p.i., all 3 tested shrimp gave moderate to strong RT-PCR product bands. No RT-PCR product was obtained at time 0 h or with normal shrimp DNA (Fig. 4). The stability of haemolymph samples was low. Fresh or Trizol-fixed haemolymph from YHV-infected shrimp gave positive RT-PCR reactions if stored for not more than 12 h at –80°C. Longer periods of storage at –80°C resulted in negative RT-PCR reactions.

The ability of RT-PCR to detect YHV in experimentally infected shrimp was compared to detection by H&E staining. The results demonstrated that RT-PCR was much more sensitive, in that YHV could be detected in all 3 of the YHV-injected shrimp by 12 h p.i. (Table 1). By contrast, H&E staining of haemolymph samples and gills revealed typical YHV histopathology only at 48 h p.i.

For a preliminary field test, haemolymph samples (10 µl) were collected from 5 shrimp randomly selected from each of 6 ponds near to a pond diagnosed as infected with YHV. The haemolymph samples from each pond were pooled (total 50 µl) and then extracted with Trizol™ reagent. The extracts were then subjected to RT-PCR using the optimized conditions described. Of the 6 ponds tested, 2 were YHV positive by RT-PCR amplification and 4 were not. Within 1 mo after sampling, all of the shrimp in 1 positive pond were lost to YHV. All the other shrimp crops were lost to YHV in the subsequent month.

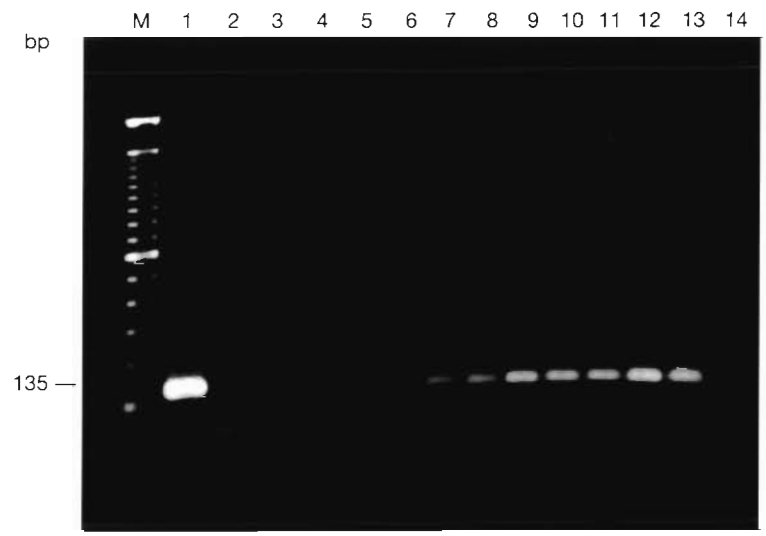

Fig. 4. RT-PCR products at various times post injection of YHV into shrimp. Ethidium bromide staining of RT-PCR products amplified at an annealing temperature of 52°C for 40 cycles using 10 ng of YHV RNA (lane 1). RT-PCR products of 3 experimentally infected shrimp at 0 h (lanes 2 to 4), 6 h (lanes 5 to 7), 12 h (lanes 8 to 10) and 18 h (lanes 11 to 13) post injection. No RT-PCR product was obtained using 10 ng of healthy shrimp DNA as the template (lane 14). Lane M contained a 100 bp size marker. The faint bands visible in lanes 2 to 4 are primer dimers

Table 1. Sensitivity of RT-PCR and H&E for detection of YHV at various times post infection. Haem: haemolymph; ND: not done

Time post injection (h)	No. exposed shrimp tested	RT-PCR positive				H&E staining positive	
		Weak	Light	Medium	Strong	Haem	Gills
0	3	0	0	0	0	0	0
6	3	1	1	0	0	0	0
12	3	0	1	1	1	0	0
18	3	0	0	1	2	0	0
24–36	9	ND	ND	ND	ND	0	0
42	3	ND	ND	ND	ND	3	0
48	3	ND	ND	ND	ND	3	3

DISCUSSION

The purpose of this work was to develop a specific and sensitive RT-PCR for the detection of YHV. If successful, the detection of YHV RNA by this process would have advantages over direct dot blot nucleic acid hybridization tests in terms of a higher sensitivity and a shorter detection time. However, the conditions have to be optimized in order to obtain the highest sensitivity and to eliminate non-specific amplification. The disadvantage is the requirement for equipment and reagents to carry out PCR amplification and agarose gel electrophoresis. On the other hand, there are alternatives which can eliminate the need for agarose gel electrophoresis detection of PCR products. These include possible modifications for rapid (30 min) membrane-based or microtiter plate-based visual detection by reverse hybridization using biotinylated dUTP incorporation during PCR amplification (see Boehringer-Mannheim catalogue). There is also a recently described experimental method for direct and rapid visual detection of hybridization which might be developed for use with normal, unlabelled PCR amplification products (Elghanian et al. 1997). These types of detection can be used to combine the sensitivity of PCR amplification and hybridization without the need for electrophoresis.

The primer pair (10F and 144R) appeared to be YHV-RNA specific, since an RT-PCR product of expected size was amplified only when nucleic acid isolated from YHV-infected *Penaeus monodon* was used as a template. The nucleic acids extracted from tissues of naturally diseased shrimp with YHV and from shrimp with experimental infections of YHV also consistently gave RT-PCR products of the same size (data not shown). As expected, no amplification product was obtained when using nucleic acids extracted from the tissues of clinically healthy shrimp and from various microorganisms.

The problem with the normal histological laboratory procedures (i.e. fixing, embedding, sectioning, staining and mounting) is that they may require several days to complete and they are too slow for farmers who need rapid confirmation in order to decide on an emergency salvage harvest before remaining shrimp die. Rapid staining of gills (Flegel et al. 1997) combined with staining of haemolymph smears (Nash et al. 1995) can speed up the diagnosis to within 3 h. However, the characteristic YHV histopathology can be seen in the gills of only moribund shrimp, so rapid staining of gills has no predictive value. The predictive value of haemolymph smears may also be low, since the laboratory tests showed that YHV histopathology could not be seen earlier than 42 h p.i., near the time of approaching morbidity. The gross signs of typical YHV infections (i.e. abnormally high feeding rates before the onset of mortality and light yellow coloration of the cephalothorax) do not help because they are not always seen in YHV outbreaks (Felgel et al. 1995). Thus, RT-PCR may be a way of solving these problems. It is very sensitive, specific, and capable of providing diagnostic results within a day. The technique may also prove useful for comparative studies of similar viruses (e.g. that described by Spann et al. 1995) and for screening of carrier shrimp larvae, carrier broodstock and reservoir hosts. However, the practicality and cost-effectiveness of RT-PCR for routine surveillance has not yet been established and, therefore, its diagnostic efficiency in field applications remains to be determined.

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