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).

[illegible] [illegible] [illegible] TGA AAA ACT ACG ACA GAA ACA
CCG GCA TGT TTT GTT CTC TCA CTG AAT TCC
AGC TCT CTC TCT CTT ACA TCC TCT ACC GTT
CTG AAG CAT ACC 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)

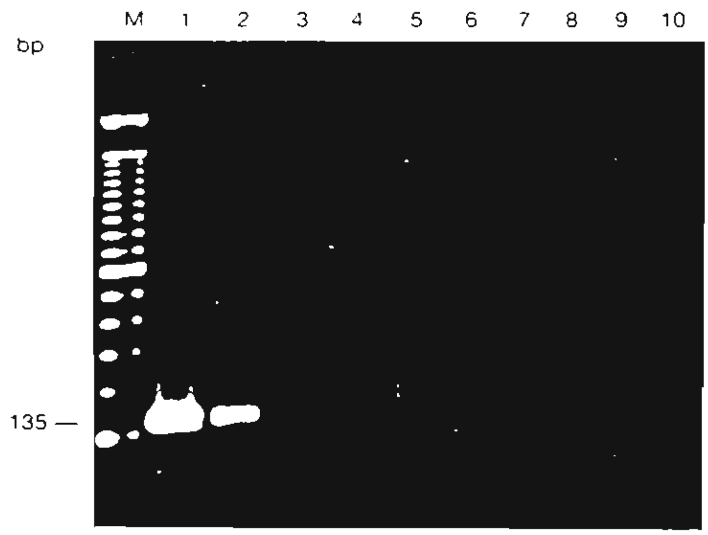

Fig. 2. Ethidium bromide staining of RT-PCR products amplified at an annealing temperature of 52°C for 40 cycles using 10 and 1 ng of YHV RNA (lanes 1 and 2), WSBV DNA (lane 3), HPV DNA (lane 4), NPV DNA (lane 5), *Salmonella* DNA (lane 6), healthy shrimp DNA (lane 7) and control template RNA from plasmid 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

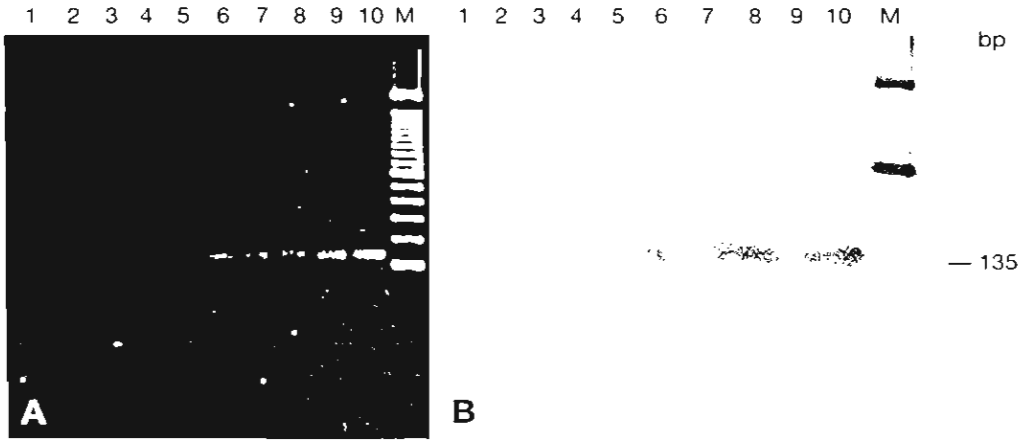

Fig. 3. Sensitivity of the RT-PCR assay. (A) Ethidium bromide staining of RT-PCR products amplified at an annealing temperature of 52°C for 40 cycles using serially diluted (1:10) YHV genomic RNA in the range of 1 ng to 0.01 fg as the template for RT-PCR (lanes 10 to 2, respectively). RT-PCR product 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.

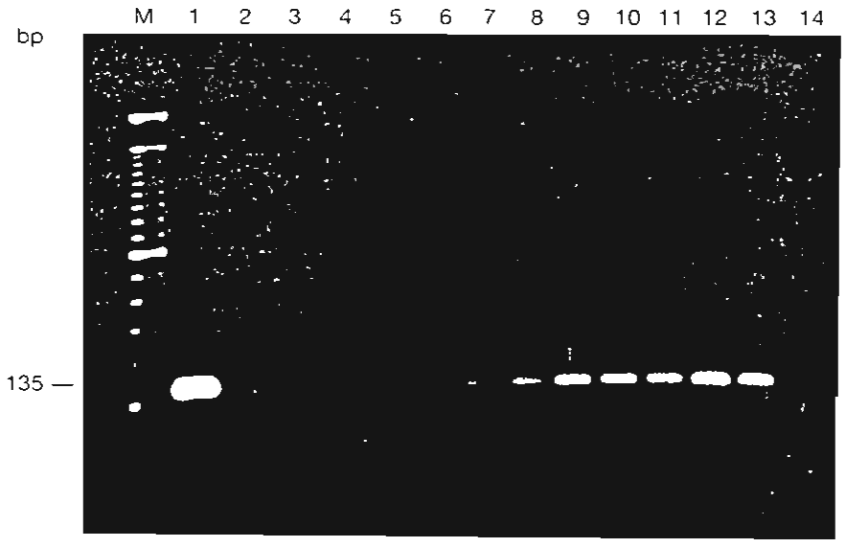

Fig. 4. RT-PCR products at various times post injection of YHV into shrimp. Ethidium bromide staining of RT-PCR products amplified at an annealing temperature of 52°C for 40 cycles using 10 ng of YHV RNA (lane 1). RT-PCR products of 3 experimentally infected shrimp at 0 h (lanes 2 to 4), 6 h (lanes 5 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	1	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 (Flegel 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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[illegible] of yellow-head virus (YHV) and the Chinese [illegible] [illegible] *Penaeus* [illegible] (Boone) [illegible] Health Section, [illegible] Society, Manila [illegible]

[illegible] rapid [illegible] diagnosis of yellow-head [illegible] Fish Health Section, [illegible] Society, [illegible]

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Experimental transmission of White Spot Syndrome Virus (WSSV) from crabs to shrimp *Penaeus monodon*

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ABSTRACT: White spot syndrome virus (WSSV) of the black tiger prawn *Penaeus monodon* is a recently discovered baculo-like virus disease which is currently the cause of very serious and widespread losses in the shrimp industry in Thailand and elsewhere in Asia. Three suspected crab carriers of this virus commonly found in shrimp-farming areas were investigated. These were *Sesarma* sp., *Scylla serrata* and *Uca pugilator*. All these crabs could be infected with WSSV by injection and they sustained heavy viral infections for up to 4 d post-injection by means of histology, specific *in situ* DNA hybridization and PCR amplification without visible signs of disease or mortality. All of them also transferred the disease to *P. monodon* via water while physically separated in aquarium cohabitation tests. Transfer of the virus to the shrimp was monitored using *in situ* DNA hybridization and PCR assay at 12 h intervals after cohabitation began. With *U. pugilator*, WSSV could be detected in the shrimp cohabitants after 24 h using PCR amplification and after 60 h using *in situ* hybridization. With *S. serrata* the shrimp were positive for WSSV after 36 h using PCR and after 60 h using PCR + *in situ* hybridization. With *Sesarma* sp. they were positive after 48 h using PCR and after 60 h using *in situ* hybridization. These laboratory trials demonstrated that crab carriers of WSSV may pose a real threat to cultivated shrimp. Most of the studies were carried out in containers with a small volume and with relatively clean sea water as compared to shrimp cultivation ponds. Pond-based studies are now needed to determine whether factors such as pond volume, pond water quality and shrimp and crab behavior can influence the rate and success of transfer.

KEY WORDS: White spot syndrome virus · WSSV · *Penaeus monodon* · *Sesarma* sp. · *Uca pugilator* · *Scylla serrata*

INTRODUCTION

Black tiger shrimp culture in Thailand has expanded tremendously in the past few years and the country has become the world's leading producer of cultivated shrimp (Rosenberry 1997). Although the industry is undergoing rapid development in a number of Asian countries, successful production is increasingly hampered by many factors, including environmental pollution, poor management and disease (Flegel et al. 1995a, b). Of the infectious diseases, bacterial and viral agents, either as single or multiple pathogens, have caused most of the production losses (Flegel 1997).

White spot syndrome virus (WSSV) presently overshadows all other disease agents as the leading cause of production losses (Flegel 1997, Flegel et al. 1997). It was first reported in Thailand as an accidental infection in laboratory-reared shrimp in early 1994 (Wongteerasu-

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paya et al. 1995), but the first farm infections were not reported until late 1994 (Wongteerasupaya et al. 1996). At that time, the virus was compared to viruses of the Baculoviridae and to the subfamily of the non-occluded baculoviruses, the Nudibaculoviridae (Francki et al. 1991). In a subsequent report of the International Commission for Taxonomy of Viruses (ICTV) (Murphy et al. 1995), this classification was canceled and the viruses were left unassigned. However, to avoid confusion, we will use the old designation (Francki et al. 1991) for WSSV as a non-occluded baculovirus. The field signs of WSSV infection are rapid and massive mortality with diseased shrimp showing white spots 5 to 6 mm or more in diameter which remain embedded in the cuticle when it is removed. The spots are sometimes accompanied by a general reddish coloration of the whole shrimp body. Since late 1994, there have been increasing numbers of field reports of massive shrimp mortality associated with WSSV in several species of penaeid shrimp (Takahashi et al. 1994, Chou et al. 1995, Durand et al. 1996, Lo et al. 1996a, Wongteerasapaya et al. 1996, Peng et al. 1997). The disease has also been detected in a wide range of wild crustaceans, including, crabs, lobsters, and shrimp (penaeid and non-penaeid) by DNA diagnostic techniques (Lo et al. 1996a, b, Maeda et al. 1997, Peng et al. 1997). However, detection alone, especially by histology and polymerase chain reaction (PCR) technology, cannot be used to confirm whether tested animals are actively infected with the virus or just mechanical carriers. *In situ* DNA hybridization tests (Wongteerasupaya et al. 1996) and studies with the electron microscope have been used to establish that some of the suspected carriers actually have active viral infections. However, even if infections are confirmed, it cannot be automatically concluded that the infected animals can transmit the virus to cultivated shrimp. Information regarding the incidence of natural carriers and the potential risk they pose is of vital importance to shrimp farmers, if they are to institute effective measures to prevent viral infections without undue expense. Unpublished studies in Thailand (B. Withyachumnarnkul) implicated several common crab species as WSSV carriers in feeding trials with shrimp. Some of the suspected species and genera corresponded to those captured and found to be PCR positive for WSSV in Taiwan (Lo et al. 1996b) and Japan (Maeda et al. 1997). In addition, Supamattaya et al. (1998) have shown that crabs can be infected with WSSV by feeding on infected shrimp tissue, by immersion in water containing viral extracts and by injection. Therefore, the purpose of this study was to determine whether 3 suspected carrier crab species commonly found in shrimp-rearing areas of Thailand could successfully transfer the viral disease to *Penaeus monodon* in laboratory tests.

MATERIALS AND METHODS

Shrimp and crab specimens. Normal *Penaeus monodon* (approximately 20 g each) were collected from a shrimp farm in Thailand. All were maintained in aerated aquaria at 30 to 35°C and fed on a dry commercial shrimp feed diet twice daily. Three adult crab species, i.e. *Sesarma* sp. (approximately 30 g each), the mud crab *Scylla serrata* (approximately 300 g each), and the fiddler crab *Uca pugilator* (approximately 30 g each), were collected from shrimp culture areas in the central region of Thailand. Crabs were maintained in aquaria under conditions similar to those for the shrimp, except that they were not completely submerged, and they were fed the same diet regimen. Haemolymph samples (50 µl for smears and 5 µl for PCR = total 55 µl) could be drawn from the shrimp or crabs at 12 h intervals without causing mortality.

***In situ* DNA hybridization.** The DNA probe for WSSV was prepared from laboratory-infected shrimp as previously described (Wongteerasupaya et al. 1995). It was non-radioactively labeled with digoxigenin using the random prime method, following the instructions accompanying the Boehringer Mannheim kit.

In situ hybridization with tissue sections was carried out as previously described (Wongteerasupaya et al. 1996). For *in situ* hybridization with shrimp and crab haemolymph, Davidson's fixative was modified by the replacement of acetic acid with distilled water. The fixative was held in a syringe at twice the volume (100 µl) of the haemolymph to be drawn (50 µl). After drawing the haemolymph, it was immediately mixed thoroughly with the fixative and then smeared on Fisher plus microscope slides (Fisher Scientific) and left to air dry. The method for *in situ* hybridization was then carried out as instructed in the Boehringer Mannheim Genius kit manual with the following modifications. Haemolymph smears were immersed in PBH buffer (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in 1 l distilled water adjusted to pH 7.4 with 1N HCl) for 5 min. They were then removed, air dried and heated at 95°C for 10 min before the addition of 20 to 25 µl probe cocktail (50× Denhart's Solution, 50% w/v dextran sulfate, 10 ng ml^{-1} sonicated salmon sperm, 20× saline sodium citrate, SSC, 50% formamide, 10 ng $µl^{-1}$ digoxigenin-labelled probe). The slides were then heated at 96°C for 6 min. After quick cooling on ice for 1 min, they were placed in a humid chamber and allowed to hybridize overnight at 42°C. Subsequent processing for blocking and detection were as described in the manual. The slides were then counterstained for 1 min with 0.5% Bismark brown, dehydrated and mounted with Permount (Fisher Scientific). There was no protease treatment step in the haemolymph protocol, since this was found to yield false negative results.

PCR amplification tests. Haemolymph samples (5 µl) were heated in 0.5 ml Eppendorf tubes at 95°C for 10 min (until dry). For amplification, 50 µl of PCR reaction mixture was added (PCR buffer pH 9.0, 1.5 mM $MgCl_2$, 1 mM of each deoxynucleotide triphosphate, 1 µM of each primer and 2 units of *Taq* DNA polymerase). PCR primers were prepared based on the sequence of a specific probe described by Wongteerasapaya et al. (1996). These primers yielded a 294 base pair (bp) fragment specific for WSSV DNA. The PCR mixture was overlaid with 50 µl of mineral oil to prevent evaporation. The reaction was then carried out for 35 cycles of denaturation, annealing, and polymerization. The first cycle included heat denaturation at 90°C for 3 min, annealing at 60°C for 30 s, and polymerization at 72°C for 30 s. The next 33 cycles used heat denaturation at 90°C for 30 s, annealing at 60°C for 30 s and polymerization at 72°C for 30 s. For the last cycle, the heat denaturation temperature was 90°C for 30 s, annealing was 60°C for 30 s, and polymerization was 72°C for 5 min. Amplified products were detected by electrophoresis of 20 µl aliquots through 1.5% agarose gel in TBE buffer.

H&E staining. Haemolymph samples were mixed with modified Davidson's fixative (2:1) and smeared on slides as described above. After air drying, they were immersed in Mayer's Haematoxylin (5 to 10 min) and then rinsed with tap water for 15 min. Next, they were stained with eosin (5 min), dehydrated with an ethanol series, transfered to xylene and mounted in permount (Fisher Scientific). Whole moribund shrimp (counted as dead when removed) and crab specimens at the end of the tests were preserved in Davidson's fixative and processed for normal histology as described by Bell & Lightner (1988).

Experimental transmission tests. Before tests were performed, haemolymph samples of experimental animals (shrimp and crab) were first assayed for WSSV by PCR assay to assure that they had no detectable infection. Preliminary tests were performed in which the crabs were experimentally infected with WSSV by injection of 100 µl of viral suspension. The viral suspension was prepared by dilution of haemolymph harvested from experimentally WSSV infected shrimp in lobster haemolymph buffer (LHB) (Boonyaratpalin et al. 1993). Injected viral suspensions contained approximately 2.7×10^4 virions as measured by standardized PCR against quantified dilutions of purified viral DNA and considering the viral genome to be 168 kbp (Wongteerasupaya et al. 1995). For each species of crab, 7 individuals were injected with WSSV and 7 were not. They were then followed for 45 d with periodic individual sampling of the haemolymph (5 µl) for PCR assays. At Day 45 they were sacrificed, fixed in Davidson's fixative and examined histologically and for WSSV.

For transmission tests, 3 experimentally infected crabs of each species (separate from the crabs described above and also verified WSBV negative by PCR) were placed (96 h post injection) in aquaria (70 × 30 × 40 cm) at 27°C with 10 uninfected shrimp (verified by PCR) but separated from them by a wire mesh. Haemolymph samples (5 µl) were drawn from each shrimp at 12 h intervals and assayed for WSSV using PCR. Mortality was recorded for both crabs and shrimp in all experiments. Controls comprised uninfected crabs cohabitant (but separated by a wire cage) in aquaria with 10 uninfected shrimp.

RESULTS

Experimental crab infections

All 3 crab species could be infected with WSSV by injection. The haemolymph of individual crabs of all 3 species was sampled every 12 h for the first 108 h, then at daily intervals thereafter for 45 d after injection. The virus was first clearly detected by means of PCR analysis of haemolymph samples from *Uca pugilator* at 24 h (Fig. 1a), but later for *Scylla serrata* (36 h; Fig. 1b) and *Sesarma* sp. (48 h; Fig. 1c). The intensity of the first visible PCR band at 48 h for *Sesarma* sp. was weak and did not photograph well. A very clear band was not evident until 72 h and onwards. The gels in the figures show the results for samples up to 96 h only, but later samples also gave PCR bands of high intensity, indicating relatively high levels of infection. In contrast to PCR, a much longer time was required before the virus could be detected by *in situ* DNA hybridization using haemolymph smears (see Table 1). Representative examples of positive and negative *in situ* hybridization reactions and histopathology by H&E staining of gills are shown in Fig. 2 for *U. pugilator* only, but results for the other crab species were similar. H&E staining of haemocytes did not reveal any differences between normal and infected crabs. No positive PCR results, *in situ* hybridization results or WSSV histopathology were obtained with the control crabs which were not injected with WSSV. There were no mortalities among

Table 1. *Uca pugilator*, *Scylla serrata* and *Sesarma* sp. Time post injection for detection of WSSV in infected crabs using 2 methods. The crab haemolymph was sampled every 12 h

Crab species	Time post injection required for detection	
	PCR assay	*In situ* hybridization
U. pugilator	24 h	60 h
S. serrata	36 h	60 h
Sesarma sp.	48 h	72 h

the control or virus-injected crabs over the 45 d observation period for these tests, although the infected crabs were PCR positive for WSSV throughout the period of observation (Fig. 3).

Transmission experiments

Shrimp kept as cohabitants with infected crabs began to die within 3 d with *Uca pugilator*, *Scylla serrata*

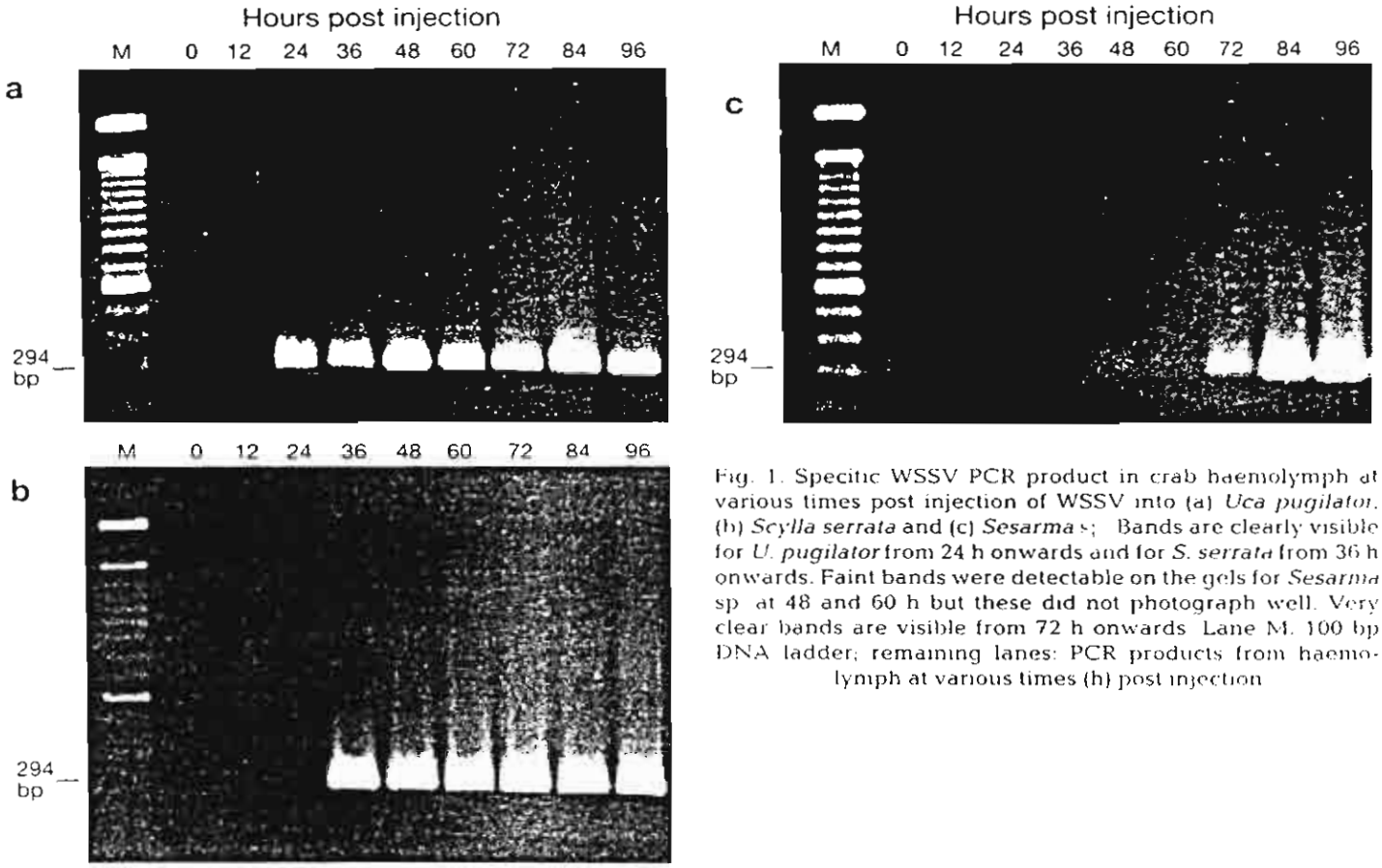

Fig. 1. Specific WSSV PCR product in crab haemolymph at various times post injection of WSSV into (a) *Uca pugilator*, (b) *Scylla serrata* and (c) *Sesarma* sp. Bands are clearly visible for *U. pugilator* from 24 h onwards and for *S. serrata* from 36 h onwards. Faint bands were detectable on the gels for *Sesarma* sp. at 48 and 60 h but these did not photograph well. Very clear bands are visible from 72 h onwards. Lane M: 100 bp DNA ladder; remaining lanes: PCR products from haemolymph at various times (h) post injection

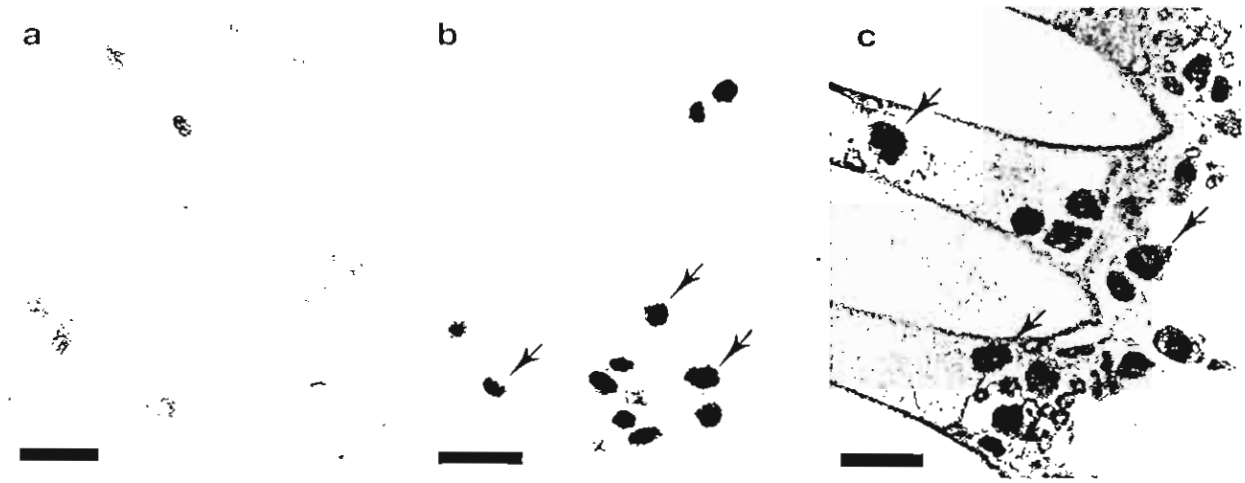

Fig. 2. *In situ* hybridization and H&E staining of tissues from *Uca pugilator* (scale bars = 15 µm). (a) *In situ* hybridization of haemolymph from normal control *U. pugilator* at 60 h, showing a negative hybridization reaction. (b) *In situ* hybridization of haemolymph from *U. pugilator* at 60 h after injection with WSSV, showing a positive hybridization reaction (dark staining nuclei, some marked by arrows). (c) H&E staining of gill tissue from *U. pugilator* at 96 h post injection with WSSV (i.e. used as a shrimp cohabitant). Hypertrophied nuclei containing basophilic inclusions typical of WSSV histopathology are clearly visible (some marked by arrows)

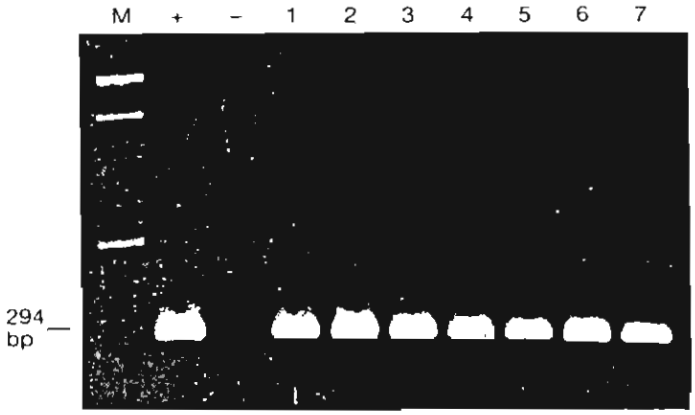

Fig. 3. Specific WSSV PCR product in haemolymph of 7 WSSV-injected *Scylla serrata* reared for 45 d. Lane M: 100 bp DNA ladder; +: positive control; –: negative control; lanes 1 to 7: individual crabs

and *Sesarma* sp. (Table 2). The presence of WSSV could be detected by normal histology and *in situ* hybridization in moribund shrimp with results similar to those shown for *U. pugilator* in Fig. 2. There was no crab mortality over the 8 d period of the test, but examination of the WSSV crabs by PCR and *in situ* hybridization during the test (data not shown) and by normal histology at the end of the test (cf. Fig. 2) showed clear evidence of WSSV infection. With both the shrimp and the crabs, no differences could be seen between haemocytes of infected and uninfected animals by H&E staining. All of the shrimp died within 8 d. There were no mortalities in the aquaria where uninfected crabs (PCR verified) were reared with uninfected shrimp (PCR verified) and all the animals remained PCR negative for WSSV throughout the period of the test. They were also histologically negative for WSSV at the end of the test.

Early detection of WSSV by PCR was possible using haemolymph from infected shrimp cohabitants (Table 2). Earliest detection was obtained when they were reared with infected *Uca pugilator* (36 h; Fig. 4a), while the time with *Scylla serrata* and *Sesarma* sp. was longer (48 h; Fig. 4b, c, respectively). Detection before the onset of mortality was possible by *in situ* hybridization, but this was usually very close to the time for onset of mortality.

Table 2. Time to detection of WSSV and time to mortality for *Penaeus monodon* reared with various WSSV infected crab species. Ten shrimp were placed in each aquarium with 3 of each test crab species. Haemolymph was sampled and tested every 12 h

Crab species	Time to detection in shrimp (h)		Shrimp mortality (no.) at days post exposure					
	PCR	*In situ*	3 d	4 d	5 d	6 d	7 d	8 d
Uca pugilator	36	60	3	4	3			
Scylla serrata	48	60	3	1	3	1	2	
Sesarma sp.	48	72	2	3	2	1	0	2

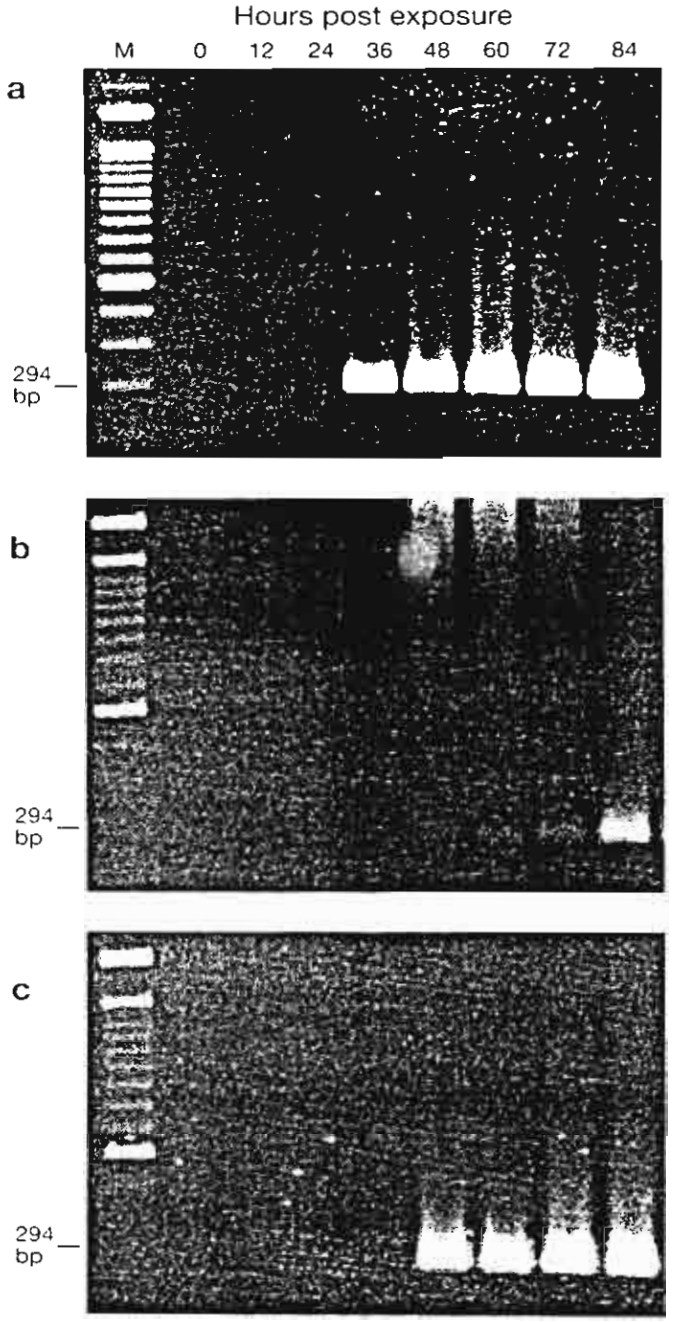

Fig. 4. Specific WSSV PCR product in haemolymph of *Penaeus monodon* at various times (h) after initiation of cohabitation with WSSV-infected (a) *Uca pugilator*, (b) *Scylla serrata* and (c) *Sesarma* sp. Clear PCR amplicons are apparent with *U. pugilator* from 36 h onwards and for *S. serrata* and *Sesarma* from 48 h onwards. Lane M: 100 bp DNA ladder; remaining lanes: PCR products from haemolymph sampled at various times (h)

DISCUSSION

The results of the experiments described here clearly show that the 3 crab species studied can be infected with WSSV by injection. Once infected, they rapidly transferred the infection to *Penaeus monodon*. This augments the work of Lo et al. (1996a, b), who reported that WSSV could be detected by PCR amplification in many crustacean carriers. The rapid experimental transmission of WSSV to *P. monodon* indicates that infected crabs in the shrimp farming environment could pose a serious threat to black tiger prawn farmers. The threat may apply to most of the shrimp cultivation industry, since WSSV also infects several other cultivated penaeid shrimp (Lightner 1996, Wongteerasupaya et al. 1996). In our study the infected crabs survived for 45 d without any mortality, showing that they can be actively infected with WSSV for extended periods without any mortality or gross signs of weakening. Supamattaya et al. (1998) also found this with *Scylla serrata*. Although the crabs in this study were infected by injection of WSSV, it has been shown that they can also be infected by immersion in WSSV contaminated sea water and by feeding on WSSV-infected shrimp (Supamattaya et al. 1998).

Because our tests were carried out in aquaria using relatively clean sea water, it is difficult to extrapolate to what might happen in an actual farm setting. Shrimp pond water contains a much more complex mixture of bacteria, phytoplankton and zooplankton than clean aquarium water. The dilution volume for viral particles escaping from a potential carrier is also much greater. These factors, along with behavior of the crabs and shrimp, would be important to consider in assessing the actual risk of viral transfer from crabs to shrimp in a real aquaculture setting. Further studies are necessary to establish the actual degree of risk. However, until there is reason to believe otherwise, the results of this study suggest that farmers would be well advised to prevent all crabs from entering their shrimp ponds throughout the cultivation cycle.

One startling feature of this work was the high degree of viremia in the crabs, as judged from the large numbers of infected cells seen in their tissues by histology and *in situ* hybridization and from the strong PCR amplification signals from their haemolymph. Yet they suffered no visible ill effects and no mortality during the period of observation. These results corresponded to those of Supamattaya et al. (1998), who also showed that crabs could carry heavy viral infections without visible negative effects. This contrasted with high mortality in *Penaeus monodon* at similar levels of viremia in the above authors' and in our tests. Clearly the crabs were not 'resistant' to the virus in the traditional sense, since they did not appear to clear it from their system or inactivate it. Rather they seemed to tolerate it at very high levels of replication. Nor did the virus appear to have lowered virulence for *P. monodon* after replication in the crabs. It is apparent that the level of viral replication, in itself, was not the cause of mortalities in the shrimp, and that some other mechanism would have to be invoked to explain it.

It is well known that insect baculoviruses process genes that inhibit apoptosis (IAP genes) (Clem et al. 1996) and that these genes allow viral replication to occur without initiating host cell death. WSSV is probably a baculo-like virus or related to the baculo-like viruses (Wongteerasupaya et al. 1995) and it may therefore contain IAP genes that function in some crustaceans but not others. Alternatively, it may be that the crabs have a high level of tolerance through prior adaptive accommodation to the same or a similar viral pathogen (Pasharawipas et al. 1997, Flegel & Pasharawipas 1998). An understanding of the reasons for differences in mortality from equally heavy viral infections may allow us to develop strategies for limiting mortality from viral pathogens in shrimp aquaculture.

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NOTE

Yellow head virus from Thailand and gill-associated virus from Australia are closely related but distinct prawn viruses

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ABSTRACT: Corresponding genomic regions of isolates of yellow head virus (YHV) from Thailand and gill-associated virus (GAV) from Australia were compared by RT-PCR and sequence analysis. PCR primers designed from sequences in the GAV ORF1b polyprotein gene amplified the corresponding 577 nucleotide region of the YHV genome. Comparison of the amplified region indicated 85.1% nucleotide and 95.8% amino acid sequence identity. YHV PCR primers designed to amplify a 135 nucleotide product previously described as a YHV diagnostic probe failed to amplify the corresponding product from GAV RNA. However, the cognate GAV sequence for this and another recently reported YHV sequence were located in an upstream region of the ORF1b gene. A comparison of these sequences indicated identities of 83.0 and 80.9% at the nucleotide level and 86.7 and 86.5% at the amino acid level, respectively. The data indicate that GAV and YHV are closely related but distinct viruses for which differential diagnostic probes can be applied.

KEY WORDS: Yellow head virus (YHV) · Gill-associated virus (GAV) · Lymphoid organ virus (LOV) · *Penaeus monodon* · RT-PCR · Sequence analysis

Yellow head virus (YHV) was first reported to be associated with mass mortalities of farmed *Penaeus monodon* (black tiger prawns) in Thailand in 1990 (Limsuwan 1991). The disease affects juvenile to subadult prawns and is usually characterised by a pale to yellowish colouration of the cephalothorax and gills, and erratic swimming of infected animals near the surface at the pond edge. Enveloped, rod-shaped virions and helical nucleocapsids are observed by transmission electron microscopy in the cytoplasm of infected haemocytes, lymphoid organ and gill cells (Boonyaratpalin et al. 1993, Chantanachookin et al. 1993). In 1994, a similar virus was identified in healthy wild and farmed *P. monodon* in Queensland, Australia (Spann et al. 1995). Virions and nucleocapsids were morphologically indistinguishable from YHV, but as they were observed exclusively within abnormal cell foci in the lymphoid organ, it was named lymphoid organ virus (LOV). In 1995-96, mass mortalities of juvenile and subadult *P. monodon* at 4 farms in north and southeast Queensland led to the identification of another similar virus (Spann et al. 1997). In this case, the lymphoid organ of diseased prawns displayed extensive structural degeneration and cellular necrosis and contained large numbers of helical nucleocapsids and rod-shaped virions that were indistinguishable from YHV. The disease was transmitted experimentally to healthy *P. monodon* and, in both naturally and experimentally infected *P. monodon*, histopathology resembled that of YHV infection. However, mortality was preceded by varying degrees of red body colouration and there was no indication of pale body colouration or yellowing of the cephalothorax as described for YHV. Unlike the non-pathogenic LOV, high infection levels were observed in gills as well as lymphoid organs and the pathogenic virus was named gill-associated virus (GAV; Spann et al. 1997). In Taiwan, a yellow head-like virus has also been identified in farmed *P. japonicus* (kuruma prawns) displaying typical signs of severe white spot disease (Wang et al. 1996). Although histopathology was characteristic of YHV, its involvement in clinical disease was complicated by the likely coexistence of white spot syndrome virus.

Little is known of the molecular structure or the relationship between these yellow head-like viruses. Analyses of purified YHV preparations have indicated that virions contain a single-stranded RNA genome (Wongteerasupaya et al. 1995) and 4 major structural proteins of ~170, 135, 67 and 22 kDa (Nadala et al.

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1997). The 135 kDa protein was shown to be glycosylated (Nadala et al. 1997). Based on a rod-shaped virion morphology similar to plant rhabdoviruses and preliminary data suggesting that the large (~22 kb) ssRNA genome may be of negative polarity, YHV has been provisionally proposed to be a member of the *Rhabdoviridae* (Loh et al. 1997, Nadala et al. 1997). However, we have recently determined the nucleotide sequence of a 9.1 kb region of the GAV genome which encodes a long open reading frame (ORF) with significant homologies in the functional domains of ORF1b polyproteins of arteri-, toro- and coronaviruses (Cowley et al. unpubl.). In addition to the ORF1b (replicase) gene, the genomic region contains a ribosomal frameshift site and intergenic promoter elements indicating that the replication mechanism employed by GAV is related to these positive-strand ssRNA viruses. In this paper, we report sequence relationships between YHV and GAV based on 3 regions of the large ORF1b gene.

Materials and methods. ***cDNA library preparation:*** A GAV cDNA library was prepared by random amplification (Froussard 1992) of a ~22 kbp dsRNA replicative intermediate purified from lymphoid organ total RNA of *Penaeus monodon* infected experimentally with GAV as described previously (Spann et al. 1997). Briefly, 200 to 400 µg total RNA isolated using TRIzol-LS™ (Gibco-BRL) was resolved in a 0.6% low melting point agarose-TAE gel containing 0.5 µg ml^{-1} ethidium bromide (Sambrook et al. 1989). A ~22 kbp dsRNA band was isolated using β-agarase (Boehringer Mannheim) digestion according to the manufacturer's instructions. dsRNA (~10 ng) was strand-separated in the presence of 150 ng uni-primer-dN_6 (5'-GCCGGAGCTCTGCAGAATTC$(N)_6$-3') (Froussard 1992) by incubation in 12 µl 8% deionised formamide in DEPC-water at 98°C for 8 min followed by rapid quenching on dry ice. Representative sequences were randomly amplified using the rPCR method described by Froussard (1992) except that 200 U Superscript II (Gibco-BRL) was substituted for 20 U AMV reverse transcriptase for first strand cDNA synthesis and that Taq DNA polymerase (Promega) was used in place of AmpliTaq (Perkin Elmer-Cetus). rPCR products were purified using a QIAquick™ column (QIAGEN) and cloned by ligation into pGEM-T Vector (Promega) and transformation of competent *E. coli* DH5-α host cells (Gibco-BRL). Plasmids containing inserts were purified from colonies selected with ampicillin, X-gal and IPTG as described by Sambrook et al. (1989).

Primers and PCR reactions: PCR primers GAV1 (5'-ATCCATACTACTCTAAACTTCC-3'), GAV2 (5'-GAATTTCTCGAACAACAGACG-3'), GAV5 (5'-AACTTTGCCATCCTCGTCAC-3') and GAV6 (5'-TGGATGTTGTGTGTTCTCAAC-3') were designed to a 781 bp cDNA clone (pG12) that contained a continuous long open reading frame (ORF) and were synthesized using an Oligo-1000 DNA Synthesizer (Beckman). Total RNA was extracted from the lymphoid organs of *Penaeus monodon* infected experimentally with YHV using TRIzol-LS™ reagent (Gibco-BRL). For RT-PCR reactions, 10 ng total RNA and 30 pmol each primer GAV5 and GAV6 were added to a reaction mixture comprising 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1 U ribonuclease inhibitor (Gibco-BRL), 2 mM $MgCl_2$, 200 µM each dATP, dTTP, dCTP and dGTP, 200 U Superscript II reverse transcriptase (Gibco-BRL) and 2.5 U Taq DNA polymerase (Gibco-BRL). The mixture was incubated in a thermal cycler using 1 cycle of 42°C for 30 min, 94°C for 2 min followed by 30 cycles of 94°C for 15 s, 52°C for 15 s and 72°C for 15 s and a final elongation step of 72°C for 5 min. For nested PCR, 0.5 µl of the primary RT-PCR was amplified with the primer pair GAV1-2 using the conditions above but with reduced (i.e. 20) amplification cycles. PCR products were resolved in a 2% agarose-TAE gel containing 0.5 µg ml^{-1} ethidium bromide and visualized using a UV transilluminator. The 618 bp YHV PCR product was purified using a QIAquick™ column (QIAGEN) and cloned by ligation into pGEM-T Vector (Promega) as described above.

Sequence analyses: Both DNA strands of the 618 bp inserts in 3 YHV PCR clones were sequenced using universal pUC forward and reverse primers, Thermo-Sequenase™ (Amersham) and automated ABI Model-377 sequencing systems (Applied Biosystems Inc.) at the Australian Genome Reference Facility, University of Queensland. Nucleotide sequence translations were performed using MacVector 3.5 software. Sequence alignments and estimations of sequence identity were conducted using Clustal W software at the Australian National Genomic Information Service at the University of Sydney. Sequences reported in this paper are deposited in the EMBL/GenBank database under Accession Numbers AF102827, AF102828, AF102829 and AF126718.

Results and discussion. The relationship between the morphologically and clinically related YHV from Thailand and GAV from Australia was determined by sequence comparison of 3 genomic regions located in the ORF1b-like gene. The first 618 nucleotide region was amplified from YHV RNA by RT-PCR using primers (GAV5 and GAV6) designed from a sequence overlapping the putative helicase domain of GAV ORF1b (Fig. 1). The authenticity of the YHV PCR product was confirmed by nested PCR amplification of an internal 317 bp product using primers GAV1 and GAV2. The nucleotide and deduced amino acid sequences of the 618 bp YHV amplicon were determined from the consensus of 3 clones and compared with the corresponding GAV sequence (Fig. 2a). In each virus,

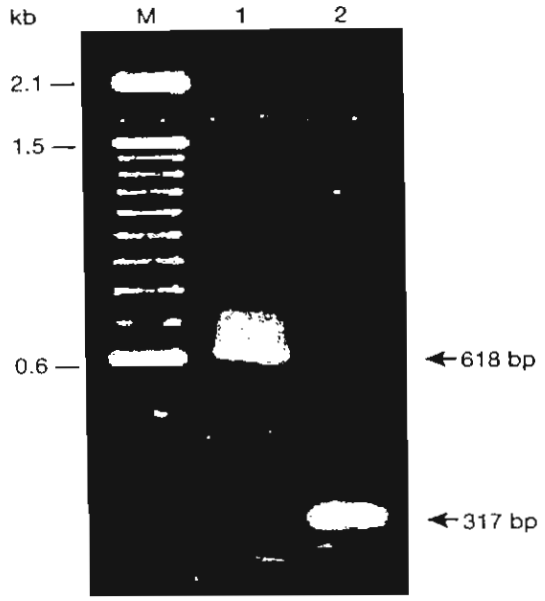

Fig. 1. Products of RT-PCR and nested PCR amplification of YHV RNA present in total lymphoid organ RNA isolated from YHV-infected *Penaeus monodon*. Total RNA (10 ng) was amplified by RT-PCR with the primer pair GAV5-6 using 30 cycles at an anneal temperature of 52°C (lane 1). An aliquot (0.5 µl) of the RT-PCR was re-amplified by nested PCR with the primer pair GAV1-2 using 20 cycles and the same conditions (lane 2). PCR products were resolved in a 2% agarose/TAE containing 0.5 µg ml^{-1} ethidium bromide. M represents a 100 bp DNA ladder marker (Gibco-BRL)

the region encodes a continuous open reading frame (ORF). In the 577 bp region internal to primers GAV5 and GAV6, there was 85.1% identity in nucleotide sequence and 95.8% identity in amino acid sequence. The majority (77/86) of nucleotide mismatches modified codons in 'wobble' positions and were silent. Most amino acid substitutions (6/8) were conservative as defined by charge and polarity conservation and matching of aromatic residues (Poch et al. 1990).

A second comparison was conducted with the GAV genomic region corresponding to the 135 bp sequence of a cDNA clone pMUY412 (Fig. 2b) used to establish a YHV diagnostic RT-PCR (Wongteerasupaya et al. 1997). In each virus, the region encodes a continuous ORF which is located towards the 5' end of the GAV ORF1b gene (Fig. 3). Nucleotide sequence identity (83.0%) was similar to that observed for the downstream 577 nucleotide region. However, amino acid sequence identity (86.7%) was somewhat lower due to a higher proportion of codon changes in the first and second base positions. Significantly, there was considerable divergence between the GAV and YHV sequences in the terminal regions of the 135 nucleotide sequence targeted by YHV RT-PCR primers 10F (6/20 mismatches) and 144R (10/20 mismatches). Four of these mismatches occur at the extreme 3'-terminus of primer 144R. As would be expected from this level of mismatch, all attempts to amplify the corresponding 135 bp product from GAV using these YHV primers have failed (data not shown).

A 1068 nucleotide region of a YHV sequence recently reported by Tang & Lightner (1999) was also analyzed. This sequence aligned with the GAV ORF1b gene upstream of and overlapping the 135 nucleotide sequence encompassed by the Thai primer pair 10F/144R (Wongteerasupaya et al. 1997). Although there appears to be an insertion error at G^{408} which results in premature termination of the reading frame, the corrected sequence (excluding the overlap) shared 80.9% nucleotide and 86.5% amino acid sequence identity to GAV (Fig. 2c). A comparison of all 3 ORF1b regions, the relative positions of which are shown in Fig. 3, indicates that the overlapping 5' regions shared similar levels of sequence identity that were somewhat lower than that identified in the 3' helicase region, particularly at the amino acid level. In the helicase region amplified by primers GAV5 and GAV6, the level of sequence divergence between YHV and GAV is similar to that observed within the same region of different strains (A59 and JHM) of murine coronaviruses (Bredenbeek et al. 1990, Lee et al. 1991). Moreover, the ORF1b gene is likely to be more highly conserved than other genes encoding structural proteins, especially in those domains of ORF1b associated with enzymatic activity.

The sequence comparisons reported here support clinical, histological and morphological observations suggesting that GAV from Australia and YHV from Thailand are closely related viruses (Boonyaratpalin et al. 1993, Chantanachookin et al. 1993, Spann et al. 1997). However, the level of sequence divergence between YHV and GAV is far greater than between individual Australian isolates of GAV which share >98.5% identity at the nucleotide level (Cowley et al. unpubl.). This indicates that YHV and GAV represent distinct genetic lineages and may be considered as different species or different geographic topotypes.

Regional and international movements of live and frozen shrimp provide opportunities for the inadvertent introduction of exotic pathogens and evidence has recently been reported for an incursion of YHV into the prawn aquaculture industry in the Americas via frozen commodity shrimp (Lightner et al. 1997, Nunan et al. 1998). Sequence analysis of yellow head-like viruses from various regions in Australasia, India and the Americas will be required to determine the extent of molecular diversity among isolates from these distinct geographical locations. Such molecular data and comparative pathological studies will be useful in epi-

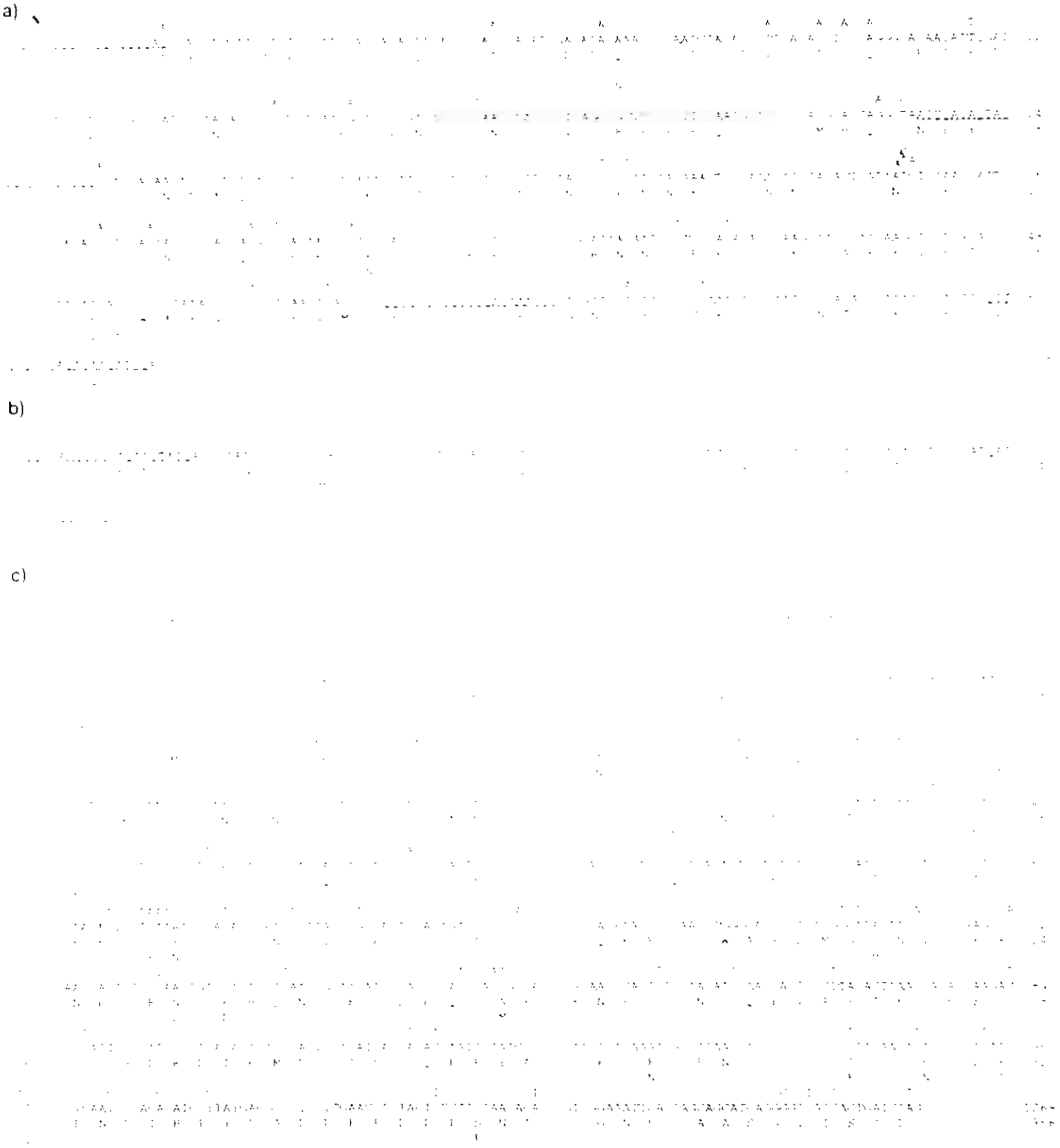

Fig. 2. Comparison of GAV ORF1b gene sequences with cognate regions of YHV including (a) a 577 nucleotide region amplified by RT-PCR with the primer pair GAV5-6, (b) a 135 nucleotide region reported by Wongteerasupaya et al. (1997) and (c) a 1068 nucleotide region of a sequence reported by Tang & Lightner (1999). The nucleotide and deduced amino acid sequences of GAV are indicated with YHV nucleotide and amino acid differences indicated above and below, respectively. YHV nucleotides not known are indicated ( ). The positions of PCR primers in (a) GAV5 (A^{1}-C^{20}), GAV6 (G^{598}-A^{618}), GAV1 (A^{230}-C^{251}) and GAV2 (C^{526}-C^{546}) and in (b) 10F (C^{2}-G^{22}) and 144R (C^{117}-T^{136}) are underlined

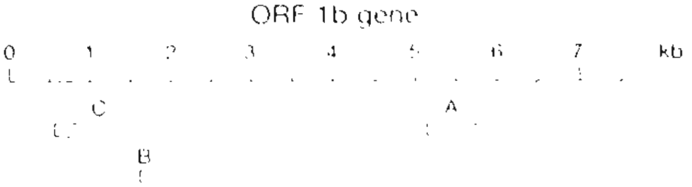

Fig. 3. Relative positions along the ~7.8 kb GAV ORF1b gene of the 3 YHV regions used in sequence comparisons. Sequence identity values (nucleotide %, amino acid %) for the regions were: (A) 572 nucleotide region (85.1%, 95.8%), (B) 1.5 nucleotide region (83.0%, 86.7%) (Wongteerasupaya et al. 1997) and (C) 1068 nucleotide region (80.9%, 86.5%) (Tang & Lightner 1999)

demiological investigations to trace possible virus sources responsible for disease episodes and to validate the origin of exotic viruses where incursions are suspected. As YHV primers 10F and 144R fail to amplify GAV sequences, they may be suitable, in conjunction with GAV-specific primers, for the differential detection of yellow head-like viruses from Thailand and Australia. Ultimately however, sequence analysis of RT-PCR products generated with generic yellow head-like virus primers will provide a more useful tool for distinguishing between isolates from different [illegible]

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