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Detection of Australian gill-associated virus (GAV) and lymphoid organ virus (LOV) of *Penaeus monodon* by RT-nested PCR

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ABSTRACT: A highly sensitive test based on reverse transcription followed by nested polymerase chain reaction (RT-nPCR) was developed to detect the Australian yellow-head-like viruses, gill-associated virus (GAV) and lymphoid organ virus (LOV) of *Penaeus monodon*. The RT-nPCR detected viral RNA in as little as 10 fg lymphoid organ total RNA isolated from GAV-infected *P. monodon*. Amplification of serial dilutions of a GAV cDNA clone showed that the nested PCR was sufficiently sensitive to detect a single genome equivalent using a DNA template. The specificity and sensitivity of the RT-nPCR was also demonstrated using experimentally infected *P. (Marsupenaeus) japonicus*, where GAV sequences could be amplified from lymphoid organ and haemocyte RNA as early as 6 h post infection (p.i.), and from gills by 24 h p.i. In contrast, transmission electron microscopy (TEM) identified nucleocapsids and virions in lymphoid organ cells and haemocytes from Days 3 and 6 p.i., respectively, while there was no evidence of infection in gill cells at any time. The practical application of the RT-nPCR was demonstrated by screening healthy wild-caught *P. monodon* broodstock. The high prevalence (>98%) of broodstock that were positive by RT-nPCR suggests that LOV is endemic in northern Queensland. In addition, results with lymphoid organ, gill and haemocyte RNA suggest that small gill biopsies may be best suited to the non-sacrificial testing of valuable broodstock. The speed and sensitivity of the RT-nPCR make it a useful adjunct to TEM for diagnosing LOV/GAV infection of *P. monodon*, with the additional benefit that screening of gill biopsies may facilitate selection of LOV-free broodstock.

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

INTRODUCTION

We have recently described a rod-shaped, gill-associated virus (GAV) which was associated with mortalities of *Penaeus monodon* prawns at 4 farms in Queensland, Australia, in 1996 (Spann et al. 1997). GAV is highly pathogenic, inducing mortalities from 4 to 5 d following experimental infection of subadult *P. monodon*. In diseased prawns, large numbers of helical nucleocapsids and enveloped virions appear in gill and lymphoid organ cells. Invariably, the lymphoid organ displays extensive cellular necrosis and structural degeneration (Spann et al. 1997). In morphology and cytopathology, GAV closely resembles yellow head virus (YHV) from Thailand (Boonyaratpalin et al. 1993, Chantanachookin et al. 1993, Wongteerasupaya et al. 1995). However, GAV does not induce the pale to yellow body colouration described for YHV, and mortality is usually preceded by a pink to red body colouration. Prior to the identification of GAV, a virus with similar morphology was reported to be common in healthy *P. monodon* in Queensland (Spann et al. 1995). Unlike GAV, lymphoid organ virus (LOV) infection appeared to be restricted to hypertrophied cells in discrete foci or 'spheroids' in the lymphoid organ. LOV is non-pathogenic, does not cause extensive cellular necrosis and has not been observed in gill tissue.

Recent molecular studies of YHV and GAV have shown that they are closely related but distinct viruses which are likely to be classified in the family *Coron-*

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aviridae (Cowley et al. 1999). Wongteerasupaya et al. (1995) demonstrated that YHV contains a single-stranded RNA genome. Nadala et al. (1997a) subsequently showed that the YHV genome is ~22 kb long and identified 4 major virion proteins (170, 135, 67 and 22 kDa) of which the 135 kDa protein is glycosylated. Tang & Lightner (1999) have amplified reverse transcription-polymerase chain reaction (RT-PCR) products from cDNA synthesised using a primer of opposite sense to a putative coding sequence to show that YHV genomic RNA is likely to be of positive polarity. We have recently determined the nucleotide sequence of the GAV genome and established that it is indeed a (+) RNA virus which contains sequence homologies and a genome organisation, including large overlapping ORF1a and ORF1b polyprotein genes translated via a −1 ribosomal frameshift site, characteristic of toro- and coronaviruses (Cowley et al. unpubl.). Comparative sequence analysis of 3 corresponding regions of the ORF1b gene has indicated that YHV and GAV are closely related viruses that may be regarded as different geographic topotypes (Cowley et al. 1999).

In this paper, we describe a sensitive and specific RT-nested PCR (RT-nPCR) for detecting GAV in infected prawn tissues. The test is applied to the detection of GAV in total RNA isolated from lymphoid organ, gill and haemocytes of healthy and clinically diseased *Penaeus monodon* and from *P. (Marsupenaeus) japonicus* infected experimentally with GAV. Sequence analysis of PCR products amplified from healthy *P. monodon* indicates that LOV is a non-pathogenic variant of GAV. In addition, screening of hatchery broodstock suggests that LOV infection may be widespread in wild *P. monodon* from northern Queensland.

MATERIALS AND METHODS

Prawns. Healthy *Penaeus monodon* and *P. japonicus* were obtained from farms and hatcheries in south-east and northern Queensland between December 1996 and March 1998. *P. monodon* broodstock were captured from coastal waters in the Cairns, Innisfail and Townsville regions of northern Queensland. The broodstock were obtained either directly from a commercial trawler operator and tested prior to spawning or from hatcheries or CSIRO Marine Research, Cleveland, and tested subsequent to spawning. Prawns were sampled within 1 to 2 d of their acquisition. Diseased *P. monodon*, displaying characteristic clinical symptoms and histological evidence of GAV infection (Spann et al. 1997), were obtained from a pond at a farm in south-east Queensland that had experienced mortalities in March 1997.

Virus infections. The prototype GAV isolate used to generate cDNA clones originated from clinically diseased *Penaeus monodon* collected from farm A in northern Queensland in 1996 (Spann et al. 1997). Healthy *P. monodon* (15 to 20 g) were injected with 5 µl g^{-1} body weight of head extracts of GAV-infected prawns as described previously (Spann et al. 1997). *P. monodon* deaths began at 4 d post infection (p.i.), and at 6 d p.i. lymphoid organ and gill tissues were isolated, snap frozen on dry ice and stored in liquid nitrogen until used for RNA isolation. Haemolymph (0.5 to 1.0 ml) was collected by cardiac puncture directly into 1 ml ice-cold modified arsenals anticoagulant (19.3 mM sodium citrate, 239.8 mM NaCl, 182.5 mM glucose, 6.2 mM EDTA, pH 7.3) (van de Braak et al. 1996). Haemocytes were collected by microcentrifugation at 5000 × *g* for 2 min, resuspended in 1 ml phosphate-buffered saline (PBS), recentrifuged and the haemocyte pellet was stored at −70°C.

To follow the progress of GAV infection in a susceptible LOV-free host, juvenile *Penaeus japonicus* (12 to 14 g) were inoculated as described above. Lymphoid organs, gills and haemocytes were pooled from 2 prawns sampled at 6, 24, and 48 h and on Days 3, 6, 9, 12 and 15 p.i. as described above and stored under liquid nitrogen until used for total RNA isolation. At the same times p.i., haemocytes and cephalothorax tissue samples from 2 prawns were also processed for histology and transmission electron microscopy (TEM).

Light and electron microscopy. Histological examinations of prawn cephalothorax tissues fixed in Davidson's fixative were conducted as described previously (Spann et al. 1997). Lymphoid organ, gill and haemocyte tissues were also fixed in modified Karnovsky's reagent and processed for TEM to confirm the presence of LOV/GAV and to quantify nucleocapsids and virions (Spann et al. 1997).

RNA isolation. In general, total RNA was extracted from 50 to 100 mg lymphoid organ, gill or haemocytes of healthy and GAV-infected prawns by homogenisation in 750 µl TRIzol-LS™ (Life Technologies) using a pellet pestle according to the manufacturer's instructions. RNA was resuspended in diethylpyrocarbonate (DEPC)-water, quantified by spectrophotometry (A_{260nm}) and stored at −70°C. All reagents and tissue samples were handled in a separate laboratory using a laminar flow cabinet and aerosol-resistant barrier pipette tips to prevent carry-over contamination with PCR products.

Random PCR amplification and cloning of GAV cDNA. Lymphoid organ total RNA was isolated from *Penaeus monodon* infected experimentally with GAV as described above. RNAs were resolved in 0.6% LMP agarose (Life Technologies)-TAE gels containing 0.5 µg ml^{-1} ethidium bromide (Sambrook et al. 1989).

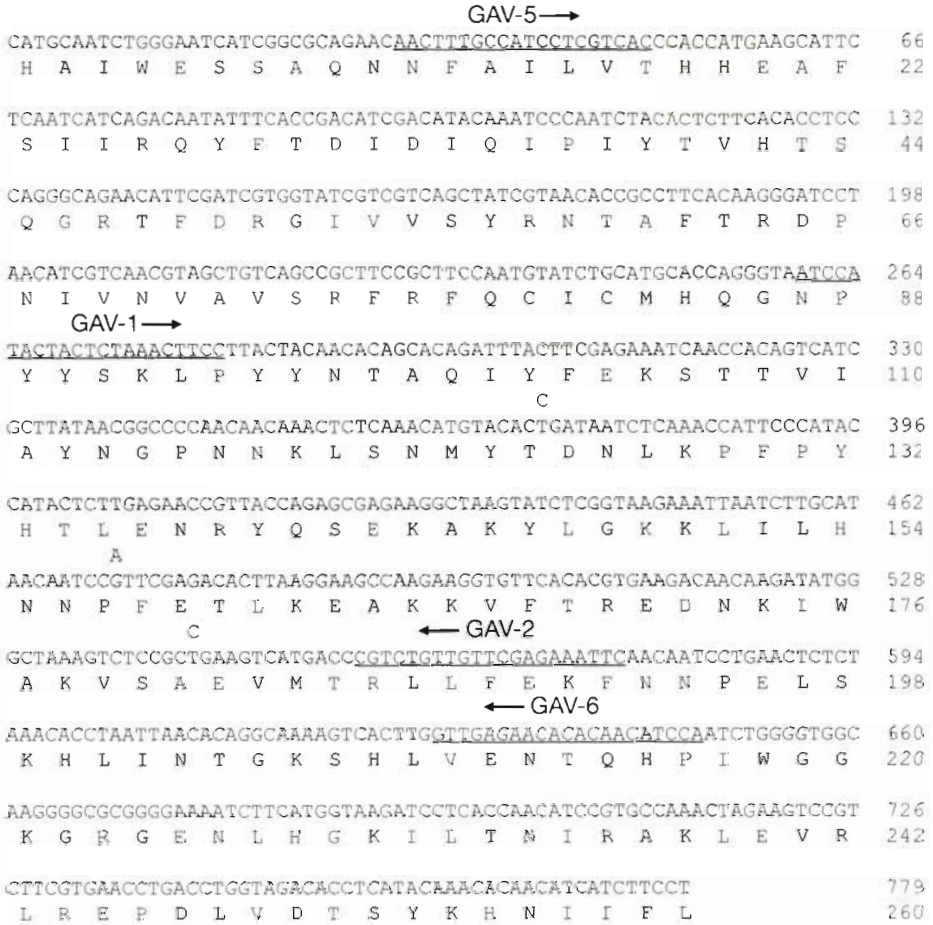

Fig. 1. Nucleotide and deduced amino acid sequence of the 781 bp GAV (gill-associated virus) cDNA clone pG12 indicating the positions of the PCR primers GAV-1 (A^{259}–C^{281}), GAV-2 (C^{566}–C^{586}), GAV-5 (A^{31}–C^{50}) and GAV-6 (G^{626}–A^{648}). Sequence variations identified in 2 clones of a 317 bp RT-nPCR product amplified from a healthy *Penaeus monodon* infected with LOV (lymphoid organ virus) (see Fig. 2) are shown above the GAV sequence

Gel purification of a ~22 kbp dsRNA and the generation of a randomly amplified cDNA library using modifications to a random PCR method (Froussard 1992) have been described previously (Cowley et al. 1999). cDNA clone pG12 was grown in TYM/ampicillin and high-purity plasmid DNA was prepared using the Plasmid Maxi Kit (QIAGEN).

PCR primers. PCR primers GAV-5 (5'-AAC TTT GCC ATC CTC GTC AC-3') and GAV-6 (5'-TGG ATG TTG TGT GTT CTC AAC-3') were designed to amplify a 618 bp region of a 781 bp GAV cDNA clone (pG12) containing a continuous open reading frame (ORF). PCR primers GAV-1 (5'-ATC CAT ACT ACT CTA AAC TTC C-3') and GAV-2 (5'-GAA TTT CTC GAA CAA CAG ACG-3') were designed to amplify a 317 bp region of pG12 internal to the region amplified by GAV-5/GAV-6. The nucleotide and deduced amino acid sequence of GAV clone pG12, which overlaps the helicase domain of a putative coronavirus-like ORF1b gene (Cowley et al. unpubl.), is shown in Fig. 1, together with the PCR primer positions. All primers were synthesised using a Beckman Model 1000 Oligonucleotide Synthesiser.

RT-PCR. Total RNA (100 ng) was denatured in the presence of 35 pmol of each primer (GAV-5 and GAV-6) by heating at 98°C for 8 min in 6 µl DEPC-water containing 0.5 µl deionised formamide and quenched on dry ice. cDNA was synthesised by the addition of 2 µl Superscript II buffer × 5, 1 µl 100 mM DTT, 0.5 µl 10 mM dNTPs, 20 U rRNasin™ (Promega) and 100 U Superscript II reverse transcriptase (Life Technologies) and DEPC-water to 10 µl, and the reaction was incubated at 42°C for 1 h followed by heating at 99°C for 5 min before quenching on ice. To determine the sensitivity of the RT-PCR, 10-fold dilutions of GAV-infected lymphoid organ RNA from *Penaeus monodon* were diluted in 100 ng μl^{-1} uninfected *P. japonicus* lymphoid organ total RNA.

One-tenth of the cDNA reaction (1 µl = 10 ng RNA) was amplified in 50 µl using *Taq* buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100), 1.5 mM $MgCl_2$, 35 pmol each primer GAV-5 and GAV-6 and 200 µM dNTPs overlaid with 50 µl liquid paraffin. PCRs were initiated using a 'hot-start' protocol (Chou et al. 1992) in which the reaction was heated at 85°C for 5 min prior to the addition of 2.5 U *Taq* polymerase

(Promega). DNA was amplified by 30 cycles of 95°C/1 min, 58°C/1 min, and 72°C/40 s, followed by 72°C/10 min final extension and 20°C hold using either a Corbett Research or Omnigene (Hybaid) thermal cycler. PCR products (10 µl) were resolved in 2% agarose-TAE gels containing 0.5 µg ml^{-1} ethidium bromide.

Nested PCR. When the result of the primary RT-PCR was negative or inconclusive, 0.5 µl of the primary PCR was amplified by nested PCR as above in a 50 µl reaction volume using primers GAV-1 and GAV-2. In some cases, 5 µl of the RT-PCR was used. Nested PCR conditions were as for the primary PCR except that the extension time was reduced to 30 s and number of cycles was reduced to 20. Nested PCR aliquots (10 µl) were analysed in 2% agarose-TAE gels. When a result was still negative, the complete RT-nPCR was repeated with up to 5 aliquots of the total RNA sample. All PCR reagents were handled in a laminar flow cabinet using aerosol-resistant tips to avoid contamination. Primary RT-PCR products were handled in a separate work area to that in which nested PCRs were performed.

Sequencing analysis. For sequencing, nested PCR products were purified using a QIAquick column (QIAGEN) and cloned by ligation into pGEM-T and transformation of competent *Escherichia coli* DH5-α host cells as described previously (Cowley et al. 1999). Plasmid DNA was prepared using a RPM kit (BIO 101) and automated sequencing of inserts was conducted using universal M13/pUC forward and reverse primers, ThermoSequenase™ dye-terminator reagent (Amersham) and an automated ABI Model-377 sequencing apparatus (Applied Biosystems Inc.) at the Australian Genome Research Facility, University of Queensland.

Gill biopsies. Six male and 4 female healthy, mature *Penaeus monodon* (28 to 35 g), which had not spawned in captivity, were collected from a hatchery in northern Queensland and held in the aquarium facility at CSIRO Tropical Agriculture, Brisbane, for 25 d. Gill biopsies were performed on 5 of the 10 prawns (3 males and 2 females) by holding the prawns firmly with the dorsal side against a flat surface and gently lifting the left gill cover to expose the 2 posterior primary gill filaments. The inner filament was removed using flame-sterilised scissors, leaving approximately 1.5 to 2 mm of the filament base. Prawns were monitored for mortality and wound healing for 5 wk. Total RNA was extracted using TRIzol-LS™ from gill biopsies: (1) pooled from the 2 females; (2) pooled from 2 males; and (3) from the remaining male. RT-nPCRs were performed and analysed as described above except that 1 µg instead of 100 ng RNA was used for cDNA synthesis.

RESULTS

Specificity of the RT-nPCR

The specificity of the RT-nPCR was determined using lymphoid organ RNA isolated from several sources including (1) healthy wild *Penaeus monodon*, (2) diseased farmed *P. monodon* and (3) *P. monodon* and *P. japonicus* infected experimentally with the prototype GAV isolate (Fig. 2). RT-PCR using primers GAV-5 and GAV-6 amplified the expected 618 bp product from *P. monodon* and *P. japonicus*, either naturally or experimentally infected with GAV. Nested PCR, using primers GAV-1 and GAV-2, readily amplified an internal 317 bp product from these primary RT-PCR products. In addition, a nested PCR product was amplified from a healthy wild *P. monodon* infected asymptomatically with LOV at a level insufficient to generate a primary RT-PCR product. Nested PCR pro-

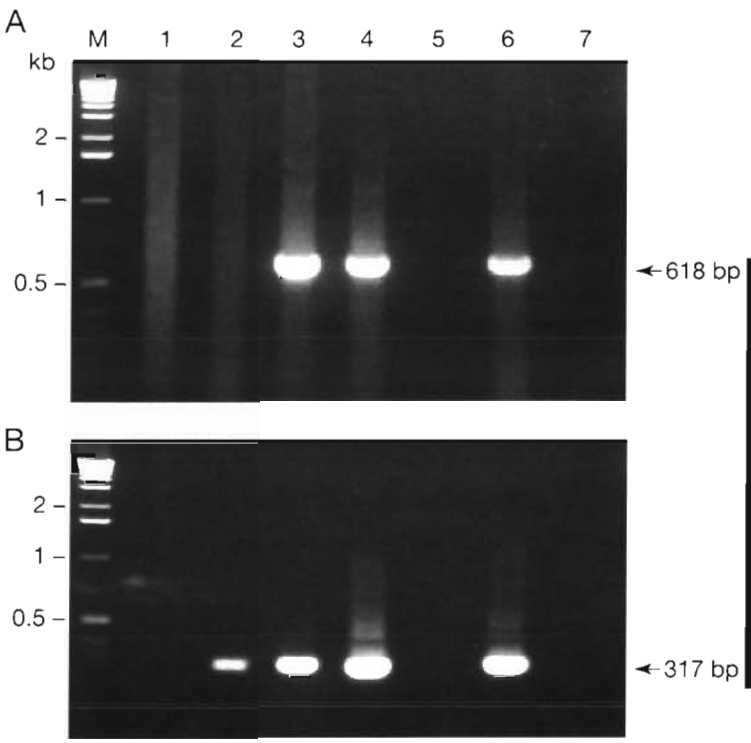

Fig. 2. RT-nPCR amplification of 10 ng lymphoid organ RNA isolated from a healthy *Penaeus monodon* (lane 1), a healthy *P. monodon* infected asymptomatically with LOV (lane 2), *P. monodon* infected experimentally with GAV (lane 3), diseased *P. monodon* from a farm outbreak of GAV (lane 4), healthy *P. japonicus* (lane 5), and *P. japonicus* infected experimentally with GAV (lane 6). A negative RT control (lane 7) was as for lane 3 with no added Superscript II RT (A) RT-PCR detection of 618 bp products, and (B) nested PCR detection of 317 bp products amplified as described in 'Materials and methods' PCR products were resolved in a 2% agarose-TAE gel containing 0.5 µg ml^{-1} ethidium bromide. M = 1 kb DNA ladder (Life Technologies)

ducts were cloned and sequenced and the sequences compared to that of pG12 generated from the 22 kbp dsRNA of the prototype GAV isolate. Sequences of 2 clones from *P. monodon* and 2 from *P. japonicus* infected with the prototype GAV were identical to the pG12 sequence. The sequences of 2 clones from the healthy *P. monodon* harbouring LOV were identical and displayed only 1.1% (3/274) nucleotide variation from pG12. None of the variations altered the coding sequence (see Fig. 1). No products were amplified from uninfected *P. japonicus* or from another overtly healthy *P. monodon*. RNA from GAV-infected *P. monodon* also failed to produce RT-nPCR products when reverse transcriptase was omitted, demonstrating that the primers were not amplifying an extraneous DNA template. In addition, the absence of RT-PCR inhibitors and the integrity of the control *P. japonicus* RNA were confirmed by RT-PCR amplification of an 848 bp fragment of 18S ribosomal RNA using primers designed for the rRNA sequence of *P. aztecus* (Kim & Abele 1990) (data not shown).

It was not possible to test for cross-reactivity with a comprehensive range of viral pathogens of *Penaeus monodon*. However, nested PCR with the GAV-specific primers failed to amplify DNA isolated from *P. monodon* postlarvae infected with monodon baculovirus (MBV) or from purified white spot syndrome virus (WSSV) (data not shown). The integrity of the MBV and WSSV DNA was confirmed using the nested PCR reported by Belcher & Young (1998) and by PCR using the PRDV P3/P4 primers reported by Kimura et al. (1996), respectively.

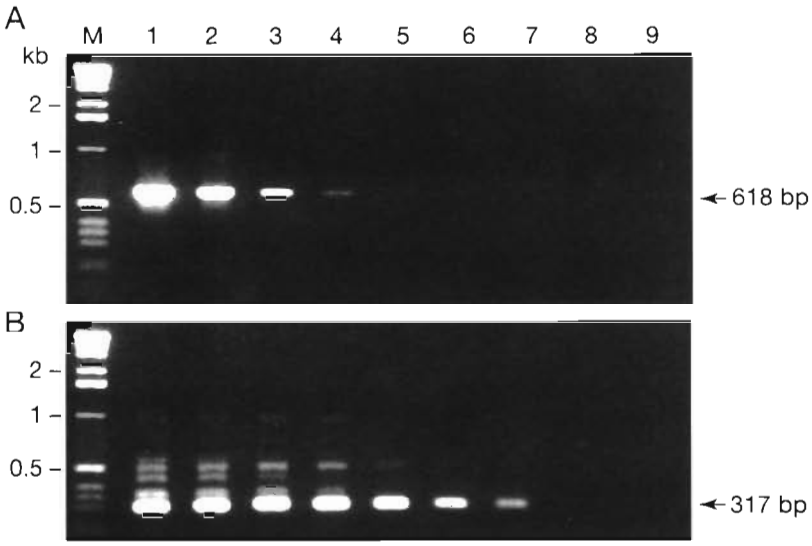

Fig. 3. Limit of detection of the primary PCR and the nested PCR. (A) PCR amplification of serial 10-fold dilutions containing 1 pg to 0.1 ag pG12 plasmid DNA using GAV-5/GAV-6 primers (lanes 1 to 8, respectively). Lane 9 represents a water control. (B) Nested PCR amplification of the primary PCRs (lanes 1 to 9) using GAV-1/GAV-2 primers. PCR products were resolved as described in Fig. 2. M = 1 kb DNA ladder (Life Technologies)

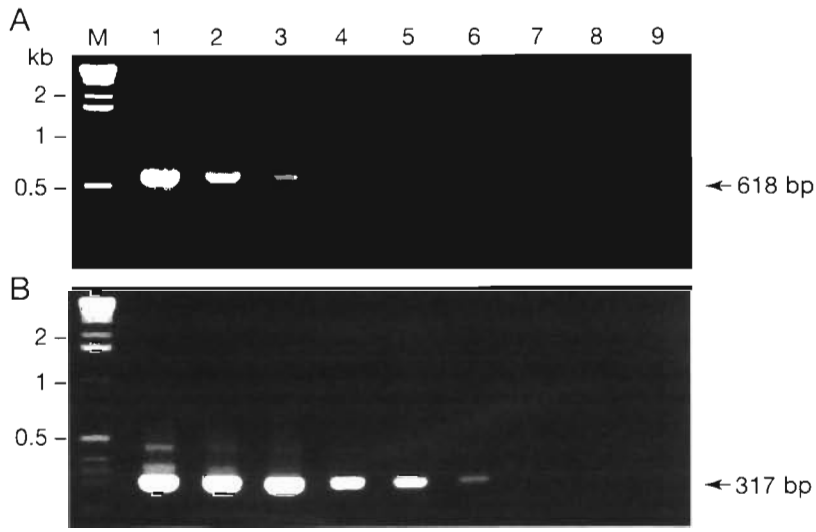

Fig. 4. Sensitivity of the RT-nPCR using a titration of lymphoid organ total RNA isolated from *Penaeus monodon* infected experimentally with GAV. (A) RT-PCR amplification of serial 10-fold dilutions containing 10 ng to 1 fg RNA (lanes 1 to 8, respectively). *P. japonicus* lymphoid organ total RNA (100 ng, lane 9) was used as a negative control. (B) Nested PCR amplification of primary RT-PCRs (lanes 1 to 9). PCR products were resolved as described in Fig. 2. M = 1 kb DNA ladder (Life Technologies)

Sensitivity and detection limit of the RT-nPCR

To determine the detection limit of the nested PCR, serial 10-fold dilutions of purified plasmid DNA (pG12, 3824 bp) were amplified in a primary PCR using the primer pair GAV-5/GAV-6, and aliquots (0.5 µl) were re-amplified in a secondary nested PCR using the primer pair GAV-1/GAV-2 (Fig. 3). Specific products were detected with 1 fg DNA (~240 genome equivalents) in the primary PCR and with 1 ag DNA (~0.24 genome equivalents) in the nested PCR. As expected, detection sensitivity could not be enhanced by increasing the amount of primary product used in the nested PCR to 5 µl.

The detection sensitivity of the RT-nPCR for viral RNA was determined using serial 10-fold dilutions of total RNA from pooled lymphoid organs of *Penaeus monodon* infected experimentally with GAV (Fig. 4). Dilutions were prepared in 100 ng μl^{-1} uninfected *P. japonicus* lymphoid organ RNA to maintain a relatively constant amount of RNA for cDNA synthesis. GAV

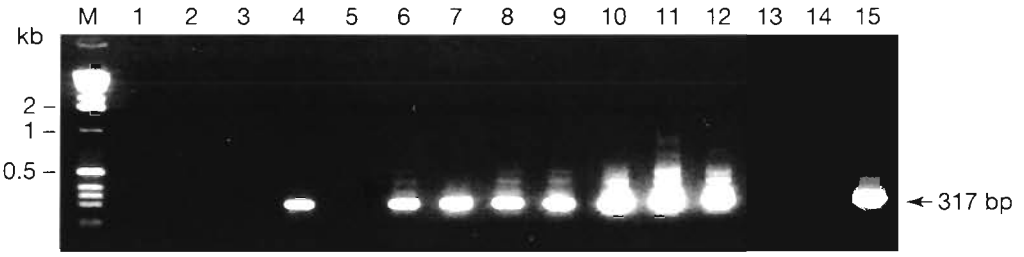

Fig. 5. Agarose gel electrophoresis of RT-nPCR products of GAV amplified from lymphoid organ (lanes 1, 4, 7, 10), gill (lanes 2, 5, 8, 11), and haemocytes (lanes 3, 6, 9, 12) of experimentally infected *Penaeus japonicus* at various times after inoculation. Amplifications were conducted using 10 ng total RNA pooled from 2 prawns sampled prior to infection (lanes 1 to 3) and at 6 h (lanes 4 to 6), 24 h (lanes 7 to 9) and 48 h p.i. (lanes 10 to 12). Controls included water (lane 13), 10 ng lymphoid organ total RNA from healthy *P. japonicus* (lane 14) and GAV-infected *P. monodon* (lane 15). The 317 bp RT-nPCR products were resolved as described in Fig. 2. M = 1 kb DNA ladder (Life Technologies)

sequences present in 100 pg total RNA were detected by RT-PCR. As observed with plasmid DNA, the nested PCR enhanced detection sensitivity by 1000-fold, allowing amplification of virus sequences from 100 fg RNA. Moreover, GAV sequences were detected in 10 fg RNA when 5 µl instead of 0.5 µl of the primary RT-PCR was used in the nested PCR (data not shown).

Comparison of RT-nPCR with TEM

To compare the sensitivity of the RT-nPCR with detection of GAV infection by histology and TEM, *Penaeus japonicus* were infected experimentally and 2 prawns were sampled at various times p.i. for lymphoid organs, gills and haemocytes. Juvenile *P. japonicus* are susceptible to GAV infection (Spann et al. unpubl.) and were used as an experimental model due to difficulties in obtaining LOV-free *P. monodon*. By RT-nPCR, GAV was detected in lymphoid organs and haemocytes from 6 h p.i. (Fig. 5). Retesting of gill RNA 3 times indicated that it was also weakly positive at this time (data not shown). At 24 h p.i., GAV was also readily detected in gill cells, and at 2 d p.i. all 3 tissue sources were clearly positive by the primary RT-PCR and yielded significantly increased amounts of the 317 bp nested PCR product. Two infected *P. japonicus* were sampled at the same time points for histology and TEM. Histological evidence of GAV infection was observed in 1 prawn on Day 6 p.i. and in all prawns sampled from Day 12 p.i. In lymphoid organ tissue, TEM identified GAV nucleocapsids and virions in ~10% of the cells on Day 3 p.i. and in up to ~30% of the cells at later time points. GAV nucleocapsids were observed in haemocytes from Day 6 p.i. but there was no evidence of GAV infection in gill cells at any stage up to 15 d p.i.

RT-nPCR screening of *Penaeus monodon* broodstock

A preliminary assessment of the usefulness of the RT-nPCR in detecting asymptomatic LOV infection in various tissues was made using 7 male and 3 female healthy wild *Penaeus monodon* broodstock that were used in captive spawnings (Table 1). The primary RT-PCR amplified LOV sequences from 10/10 prawns using lymphoid organ RNA, from 9/10 prawns using gill RNA and from 4/9 prawns using haemocyte RNA. Nested PCR amplification of the primary RT-PCRs confirmed the positive status of equivocal (+/–) results but revealed no additional positives.

RT-nPCR screening of lymphoid organ RNA from individual prawns was undertaken to determine the prevalence of LOV/GAV in *Penaeus monodon* broodstock sourced from northern Queensland, Australia. The results of testing broodstock originally captured from coastal waters in the Cairns, Innisfail and Townsville regions between September 1997 and March 1998

Table 1. RT-nPCR detection of LOV infection in wild-caught *Penaeus monodon* broodstock. +: 618 bp product amplified by RT-PCR with primers GAV-5 and GAV-6; 317 bp product amplified by nPCR with primers GAV-1 and GAV-2. +/–: weakly positive in some cases in a background of non-specific PCR products. ND: not done

Prawn	Sex	Lymphoid organ		Gill		Haemocytes	
		RT-PCR	nPCR	RT-PCR	nPCR	RT-PCR	nPCR
1	M	+	ND	+	+	+/–	+
2	F	+	ND	+	+	–	–
3	F	+	ND	+/–	+	–	–
4	M	+	ND	+	+	+	+
5	M	+	ND	+/–	+	–	–
6	M	+	ND	+/–	+	–	–
7	F	+	ND	+	+	+	+
8	M	+	ND	+	+	+/–	+
9	M	+/-	+	+/–	+	–	–
10	M	+	ND	–	–	ND	ND
Total		10/10		9/10	9/10	4/9	4/9

Table 2. Prevalence of LOV/GAV in *Penaeus monodon* broodstock determined by RT-nPCR testing of lymphoid organ RNA. Wild source: hatchery records indicated that prawns were captured from the (A) Innisfail, (B) Cairns and (C) Townsville regions of northern Queensland, Australia

Wild source	Hatchery	Date	Broodstock tested	RT-nPCR positive
A	CSIRO	25 Sep 1997	10	10
A	Hatchery 1	13 Oct 1997	12	12
B	Hatchery 2	14 Oct 1997	12	12
C	Hatchery 3	27 Mar 1998	12	12
A	Trawler[a]	5 Mar 1998	12	11
Total			58	57

[a]Prawns supplied direct from a trawler

are shown in Table 2. Of 58 broodstock tested, including the 10 reported above, 57 (98%) were positive by RT-nPCR.

Clinical effects and RT-nPCR of gill biopsies

The effects of collecting gill biopsies for RT-nPCR screening for LOV/GAV was assessed by monitoring wound healing and mortality in 5 adult *Penaeus monodon*. Melanisation of the gill cover at the point where it was lightly bent to gain access to the posterior gill filaments was observed 4 d after biopsy and persisted for the 5 wk prawns were maintained. No melanisation was observed at the biopsy site. One of the male prawns from which a biopsy was taken died during the 5 wk, as did 1 of 5 non-biopsied controls. Gill tissue pooled from the 2 female and 2 of the 3 male prawns tested positive by RT-PCR (Fig. 6). Nested PCR indicated that low levels of LOV were also present in the gills of the single male that was negative in the primary RT-PCR.

DISCUSSION

The RT-nPCR test described here provides a rapid, specific and highly sensitive method for detecting GAV in prawn tissue. The RT-nPCR also amplifies a product from *Penaeus monodon* asymptomatically infected with LOV. Sequence analysis of the 274 nucleotide internal region of the nested PCR product from LOV-infected prawns indicated 98.9% identity to the prototype GAV sequence. The 3 nucleotide differences in this region did not change the corresponding amino acid sequence. More extensive sequence comparisons, including 3 additional GAV isolates and >20 LOV isolates from healthy prawns collected from the wild in different locations in northern Queensland, have identified similar high levels of sequence identity (Cowley et al. unpubl.). These findings strongly suggest that GAV and LOV represent variants of the same virus. As we have reported previously, this level of sequence homology contrasts with the 76.3% (209/274) nucleotide sequence identity in the same genomic region of GAV and a Thai YHV isolate, which represent distinct but closely related topotypes (Cowley et al. 1999).

Analysis of many captured and farmed *Penaeus monodon* infected asymptomatically with LOV has indicated that the quantities of the 618 bp RT-PCR product can sometimes match levels obtained from GAV-infected diseased prawns (Cowley et al. unpubl.). However, some prawns are infected with LOV at levels below the detection limit of the primary RT-PCR. Nested amplification of an internal 317 bp product provides additional specificity and enhanced sensitivity. The nested PCR is 10^3- to 10^4-fold more sensitive than the primary PCR and can detect viral RNA in as little as 10 fg of total lymphoid organ RNA. Amplification of GAV sequences from plasmid DNA indicates that the nested PCR will detect the theoretical limit of a single molecule. However, competitive RT-PCR methods using synthetic RNA 'mimics' will be required to accurately quantify the detection limits for viral RNA targets. The sensitivity of the RT-nPCR is also illustrated by its ability to detect GAV RNA in lymphoid organ, gill and haemocytes within 6 to 24 h following experimental infection of *P. japonicus*. This

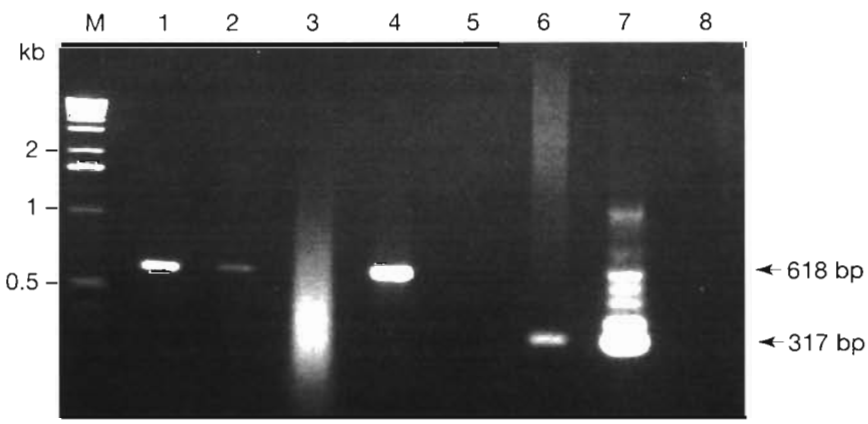

Fig. 6. Agarose gel electrophoresis of RT-nPCR products amplified from gill biopsies of adult *Penaeus monodon* infected asymptomatically with LOV. RT-PCR (lanes 1 to 5) and nested PCR (lanes 6 to 8) employed 100 ng total RNA isolated from tissue pooled from 2 females (lane 1) and 2 males (lane 2) and from a single male prawn (lanes 3 and 6). Lymphoid organ total RNA (100 ng) from an LOV-infected *P. monodon* (lanes 4 and 7) and water (lanes 5 and 8) were used as controls. PCR products were resolved as described in Fig. 2. M = 1 kb DNA ladder (Life Technologies)

is well before pathology is obvious by histological examination or viral nucleocapsids can be observed by TEM.

An RT-PCR test detecting YHV has recently been described (Wongteerasupaya et al. 1997). A 135 bp PCR product could be amplified from 10 fg purified YHV RNA which, based on a genome length of ~22 kb, represents ~820 genome equivalents. The detection sensitivity of the PCR was enhanced 1000-fold by Southern blotting using a digoxigenin-labelled DNA probe. It is unlikely, however, that this procedure would be used in routine diagnostic situations. The data reported here for GAV suggest that similar gains in sensitivity would be achieved by secondary amplification. However, the small size of the primary YHV product may restrict its usefulness as a template for nested PCR. Other *in vitro* diagnostic methods reported recently for YHV include immunological detection of viral proteins in infected prawn haemolymph by nitrocellulose-enzyme immunoassay (Lu et al. 1996) or Western blotting (Nadala et al. 1997b) using polyclonal YHV antisera. It is not yet known if LOV and GAV are sufficiently related to YHV to be detected by these tests. A non-radioactive *in situ* hybridisation test has also been developed to detect YHV in infected prawn tissues (Tang & Lightner 1999). The level of sequence identity between GAV and YHV in the region used as a probe suggests that this test may be adapted to detect GAV infections (Cowley et al. 1999).

We have recently demonstrated that both the primary and nested GAV PCR primers will amplify a product from a YHV isolate from Thailand (Cowley et al. 1999). Sequence comparisons of this and 2 other corresponding regions of the ORF1b replicase gene indicate that GAV and this Thai YHV isolate represent distinct geographic topotypes. We have also reported that there are multiple sequence mismatches between GAV and YHV in the regions targeted by YHV primers (10F/144R) amplifying the 135 bp product and that these primers do not amplify GAV RNA. Thus the GAV-5/GAV-6 primers, in combination with the YHV 10F/144R primers, may be useful in distinguishing yellow-head-like viruses from Australia and Thailand. There is a lack of information, however, on the YHV sequence targeted by the GAV-5/GAV-6 primers, and significant (14.7%) nucleotide sequence divergence exists in the amplified region. We suggest that the GAV RT-nPCR should be applied cautiously for detecting YHV, as other isolates may vary in sequence from the Thai strain. Ultimately, as more sequence data become available, RT-PCR primers could be tailored for group-specific detection or for detection of variants from different locations. Such refined PCR tests may also be useful in differentiating pathogenic and non-pathogenic variants of GAV and YHV, and in determining the origin of yellow-head-like viruses associated with disease outbreaks.

The GAV RT-nPCR is currently being applied to monitor the prevalence of LOV infection in *Penaeus monodon* broodstock captured from coastal waters in northern Queensland and to screen other common prawn species. In the 1997-1998 summer grow-out season, 57/58 (>98%) of lymphoid organ RNA samples from healthy broodstock tested positive by PCR, suggesting that LOV is endemic in wild fisheries in northern Queensland. As the majority (45) of prawns were sourced from hatcheries subsequent to spawning, it is possible that the high infection rate was attributable to their maintenance in close confinement and/or spawning in captivity. However, the detection of LOV in 11 of 12 broodstock supplied directly from a commercial trawler operator does not lend support to the supposition that significant new infections occurred in the hatcheries.

As nested PCR tests are extremely sensitive, false positives may occur if due care is not exercised in both sample processing and test implementation. We have adopted practices that (1) minimize the likelihood of sample contamination, (2) monitor for potential false positives by the routine processing of mock negative samples and (3) maximize the chances of detecting low virus loads by repeated testing of RNA from negative prawns. Although only the final data are reported, several prawns sourced from northern Queensland were negative in the initial RT-nPCR but positive in subsequent analyses, suggesting they were infected at very low levels approaching the sensitivity limit of the test. It is unlikely that such infections would be apparent by diagnostic histology or TEM. Although such methods were not applied to all broodstock tested by RT-nPCR, ad hoc screening over several seasons (Spann unpubl.) corroborates our molecular data that LOV is highly prevalent in wild and farmed *Penaeus monodon* in Queensland. Further research will be required for direct correlations of the sensitivity and reproducibility of available diagnostic tests for LOV/GAV.

There is currently little data for yellow-head-like viruses on potential horizontal transmission during fertilization/spawning or vertical transmission from broodstock to larvae. In Thailand, YHV screening of *Penaeus monodon* broodstock by TEM has suggested that post-larval infection does not constitute a major route by which disease enters farms (Flegel et al. 1997a,b). In Queensland, however, the close genetic relationship between LOV and GAV suggests a linkage between the high prevalence of asymptomatic LOV infection and outbreaks of GAV-related disease in farmed *P. monodon*. As the majority of broodstock utilised by hatcheries originates from wild stocks collected from only 2 locations, Innisfail and Cairns, in

northern Queensland, this would have significant ramifications for the industry. The RT-nPCR test will assist in addressing the important issues relating to vertical transmission of LOV/GAV and in identifying LOV-free populations of *P. monodon* that may exist at other locations in northern Australia.

The discovery that 9 out of 10 gill RNA samples from healthy *Penaeus monodon* tested positive by RT-nPCR contrasts with the complete lack of TEM evidence of LOV infection in gill tissue (Spann et al. 1995, 1997). LOV may be present at very low levels either in very few gill cells or in haemolymph or circulating haemocytes that infiltrate the tissue. Alternatively, LOV may establish a persistent infection similar to that reported recently for a mammalian coronavirus (mouse hepatitis virus) in which low levels of viral RNA replication occur in the absence of infectious virus (Bergmann et al. 1998). We have also found that the RT-nPCR can detect LOV RNA in single gill filaments biopsied from healthy adult *P. monodon*. Moreover, the prawns appear not to be adversely affected by the biopsy. Although effects on spawning efficiency need to be determined, the good correlation between detection of LOV in lymphoid organs and gills suggests that RT-nPCR testing of gill biopsies may find use in screening broodstock prior to spawning or in programs to establish specific pathogen-free (SPF) hatchery stocks.

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