

Quantitative real-time RT-PCR demonstrates that handling stress can lead to rapid increases of gill-associated virus (GAV) infection levels in *Penaeus monodon*

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ABSTRACT: Gill-associated virus (GAV) of the black tiger prawn *Penaeus monodon* has been implicated as a cause of periodic production losses in Australia since 1996. We report here the development of a real-time quantitative RT-PCR (qRT-PCR) for GAV. A dilution series of *in vitro* transcribed RNA was used to determine the sensitivity limit of the qRT-PCR and as a standard for GAV quantification. A linear relationship between cycle threshold (Ct) values and input RNA was obtained over a wide concentration range between 4.86×10^9 and 0.5 template copies per reaction, the latter being the test detection limit. The qRT-PCR was used to follow the progression of GAV levels in a group of 15 adult male *P. monodon* with chronic GAV infections that were super-infected by intramuscular injection of an inoculum containing high levels of GAV. By Day 9 post-injection, cumulative mortalities reached 100% (15/15) in the GAV-injected prawns and 40% (2/5) in placebo-injected prawns. Spermatophores were collected at the beginning, and together with other tissues, at the end of the trial. Prawns were also bled at regular intervals to collect circulating haemocytes. The qRT-PCR revealed that GAV loads increased significantly in haemocytes collected from both the control and super-infected prawns ($p = 0.010$). This increase was significantly higher in the super-infected prawns ($p = 0.047$). The rapid increase in GAV levels in super-infected *P. monodon* was expected. However, the increase in the control prawns was not, and indicates that repetitive bleeding and handling stress can stimulate GAV proliferation in chronically infected *P. monodon*.

KEY WORDS: *Penaeus monodon* · Gill-associated virus · Quantitative RT-PCR (qRT-PCR)

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INTRODUCTION

Gill-associated virus (GAV) is a virulent pathogen of black tiger prawns *Penaeus monodon* that appears to have been responsible for periodic loss in production on Australian farms since 1996 (Spann et al. 1997, 2000). Chronic GAV infection is almost universally present in wild and farmed *P. monodon* along the east coast of Australia (Spann et al. 1995, Walker et al. 2001, Cowley et al. 2002a) and is likely to be perpetuated by vertical transmission of the virus. In chronic

infections, the virus, which was originally reported as lymphoid organ virus (LOV), causes no discernible clinical signs and is restricted primarily to spheroid bodies within the lymphoid organ (Spann et al. 1995, 2003). The acute GAV infection state is associated with morbidity and mortalities and is characterised by the expansion of spheroid numbers and extensive necrosis in the lymphoid organ, the presence of virus throughout the lymphoid organ and other tissues of mesodermal and ectodermal origin, and by pathology in the eye and nerve tissues (Smith 2000, Spann et al. 2000,

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2003, Tang et al. 2002, Callinan et al. 2003). Sequence comparisons of viruses from many healthy and moribund *P. monodon* indicate that they are genetically inseparable and are now regarded as the same virus (Cowley et al. 2000 and unpubl. data).

GAV is a positive-strand ssRNA nidovirus closely related to the yellow head virus (YHV) from Thailand in genome sequence and organisation (Cowley et al. 1999, Cowley & Walker 2002). GAV and YHV have recently been classified in a new genus (*Okavirus*) and family (*Roniviridae*) within the *Nidovirales* (Mayo 2002). Both viruses have rod-shaped enveloped particles and cause similar histopathology and cytopathology (Spann et al. 1995, 1997, 2003, Tang & Lightner 1999, Tang et al. 2002). GAV diagnosis based on clinical signs and histopathology is not particularly specific as other viruses can cause similar effects. More precise diagnostic methods include transmission electron microscopy (TEM), *in situ* hybridisation (ISH) (Tang et al. 2002) and RT-nested PCR (Cowley et al. 2000). There is no detailed quantitative data directly linking GAV infection levels to the manifestation of disease symptoms. However, histological and ISH data on healthy and moribund *Penaeus monodon* and *P. esculentus* experimentally infected with GAV has recently shown that disease onset is dose-related and involves the systemic distribution of virus in connective tissues throughout the cephalothorax (Spann et al. 2003).

Real-time quantitative RT-PCR (qRT-PCR) allows the accurate quantification of viral RNA copies, and thus infection levels, by measuring the increase in fluorescence during each successive amplification cycle. This fluorescence is released by a fluorogenic (TaqMan) probe hybridised to the internal portion of the template DNA (de Wit et al. 2000, Roberts et al. 2000, Tang & Lightner 2001). As qRT-PCR requires a small amount of RNA template (Dhar et al. 2002, Durand et al. 2003), it is possible to collect a series of blood or biopsy samples and to monitor changes in viral concentration in individual prawns over time. Here we report a qRT-PCR assay for GAV and its application to monitor changes in virus levels both in naturally and experimentally infected *Penaeus monodon*.

MATERIALS AND METHODS

Development of a real-time quantitative RT-PCR assay. Oligonucleotide primers and fluorescent dye FAM-labelled TaqMan probe were designed from the GAV (pG12) sequence in Cowley et al. (2000) using Primer Express Software (PE Biosystems) default parameters. The primers GAVQPF1 (5'-GGGATCC-TAACATCGTCAACGT-3'), GAVQPR1 (5'-AGTAG-TATGGATTACCCTGGTGCAT-3') and FAM labelled

TAMRA GAVprobe1 (5'-6FAM-TCAGCCGCTTC-CGCTTCCAATG-3') were designed to amplify an 81 bp region of the GAV pG12 clone corresponding to ORF1b gene region near the C-terminus of the conserved helicase domain (Cowley et al. 2000). Optimisation of the qRT-PCR method was performed according to the ABI Prism 7700 Sequence Detection System, User Bulletin No. 2, selecting the conditions that resulted in lowest cycle threshold (Ct) and maximum ΔRn values.

Amplification reactions were performed in a volume of 25 μ l, using the TaqMan[®] Universal PCR Master Mix, No AmpErase[®] UNG, 0.3 μ M of each primer and 0.25 μ M of TaqMan probe. Amplifications (activation of the AmpliTaq for 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C) were performed using the ABI Prism[®] 7700 Sequence Detection System (PE Applied Biosystems).

To synthesise the control RNA, pG12 plasmid DNA (pGEM-T vector) containing the GAV cDNA insert (Cowley et al. 2000) was prepared from recombinant DH5 α *Escherichia coli* cells. Plasmid DNA was isolated from an overnight selective culture using the QIA-prep Spin Maxiprep system (Qiagen). The plasmid was linearised with *Nco*I (New England Biolabs), and purified by phenol/chloroform extraction and ethanol precipitation (Sambrook et al. 1989). Single stranded (+) RNA transcripts of 912 nucleotides were generated using the Ribomax-Large Scale RNA production System-SP6 (Promega) according to the manufacturer's instructions. Following transcription, DNA was removed using DNA-free[™] DNase I (Ambion), extracted with phenol/chloroform and isopropanol precipitated (Sambrook et al. 1989). *In vitro* transcribed RNA was quantified in triplicate on a GeneQuant spectrophotometer (Amersham Pharmacia) and the A₂₆₀ nm absorbances were used to determine RNA copy numbers.

Three independent serial dilutions of GAV pG12 *in vitro* transcripts (range 4.86×10^9 to 0.05 template copies per qRT-PCR reaction) were diluted in 1 μ g total RNA isolated from *Fenneropenaeus merguensis*, which is known not to be a natural carrier of GAV. These 3 independent serial dilutions were reverse-transcribed using 200 U of Superscript III Reverse-Transcriptase (Invitrogen), 40 U of RNaseOUT (Invitrogen) and 2.5 μ M random hexamer primers in a 20 μ l reaction volume according to manufacturer's instructions. These cDNAs were subsequently amplified in duplicate using the GAV qRT-PCR assay. The highest and lowest Ct values for each dilution were discarded and the standard curve generated by plotting the remaining 4 Ct values against the logarithm of the initial RNA copy numbers.

To compare the sensitivity and specificity of the qRT-PCR assay vs conventional nested RT-PCR targeting the same region of the GAV genome, cDNAs from the

serial dilutions were amplified as described in Cowley et al. (2000) with some modifications. The random-primed cDNA produced as described above was diluted 10-fold and 1 μ l was used as a template in the PCR (20 μ l reaction volume) and 1 μ l of the first step PCR was used in the nested PCR (20 μ l reaction volume). The PCR products were resolved in a 1% agarose-TAE gel containing 0.5 μ g ml⁻¹ ethidium bromide.

Quantitative detection of GAV in experimentally inoculated prawns. Preparation of the viral inoculum: Juvenile *Penaeus monodon* from the AIMS hatchery at Cape Ferguson (Queensland, Australia) displaying characteristic clinical signs of acute GAV infection were harvested and their lymphoid organ and gills collected, snap-frozen in liquid nitrogen and stored at -80°C. High levels of GAV in those tissues were verified through RT-PCR as previously described (Cowley et al. 2000). Inoculum was prepared as described by Vidal et al. (2001) with some modifications. The selected GAV positive lymphoid organs and gills were diluted 6-fold in a solution of 0.9% NaCl and homogenised. The homogenate was centrifuged at 6000 \times *g* for 15 min, the supernatant transferred to a new tube, centrifuged at 10 000 \times *g* for 20 min and the supernatant filtered to 0.2 μ m to remove bacterial contamination. Aliquots of the filtrate were stored at -80°C and tested for GAV by RT-PCR. Finally, the infectivity of the inoculum was verified by injecting 20 μ l into the ventro-lateral region of the 4th abdominal segment of 40 healthy *P. monodon* juveniles (0.99 \pm 0.51 g average weight). A control group was injected with the same volume of sterile 0.9% saline solution.

Determination of GAV concentration in viral inoculum: Total RNA from 10, 20, 40, 60, 100, 200, and 400 μ l of viral inoculum was isolated and quantified as described below. The RNA extraction efficiency was determined by plotting the total RNA yield against the extraction volume. The linear regression of this plot was used to calculate the amount of total RNA μ l⁻¹ of viral inoculum.

Absolute quantification of the number of GAV RNA template copies ng⁻¹ total RNA was determined by parallel qRT-PCR amplification of the cDNA from the inoculum with the pG12 RNA standard curve.

Experimental infection and sample preservation. Twenty wild-caught *Penaeus monodon* male adults (average weight 62.2 g) were distributed in 4 circular 1000 l tanks supplied with continuous flow-through seawater. After an acclimation period of 48 h, spermatophore and haemolymph samples were taken from all animals, snap-frozen in liquid nitrogen and stored at -80°C. Haemolymph (200 μ l) was collected by pericardial sinus puncture and placed directly into 500 μ l of ice-cold RNase-free (DEPC-treated) anticoagulant solution (Vargas-Albores et al. 1993). Haemocytes were

pelleted by haemolymph microcentrifugation at 4000 \times *g* for 4 min, snap-frozen in liquid nitrogen and stored at -80°C. Spermatophore samples were collected by electrically induced ejaculation (Sandifer et al. 1984). After the haemolymph and spermatophore collection, 15 prawns from 3 tanks were super-infected with GAV by injection of 200 μ l of viral inoculum. As a stressed non-inoculated placebo treatment, 5 prawns in the remaining tank were injected with a sterile 0.9% NaCl solution. Presently, there are no certified GAV-free stock in Australia and therefore a 'true' GAV negative control was not available in this study. All 20 prawns were bled again 4, 6, 8 and 9 d after the injection, and the haemocyte samples stored at -80°C. All animals were continuously monitored and any moribund prawns were bled and dissected. Tail muscle, sperm, lymphoid organ, hepatopancreas, and gill samples were snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction.

RNA extraction, cDNA synthesis and GAV quantification. Prawn tissues were homogenised in TRIzol-LS™ (Life Technologies) and RNA was isolated according to the manufacturer's instructions. The RNA was DNase treated using DNA-free™ (Ambion®) according to the manufacturer's instructions, ethanol precipitated and quantified spectrophotometrically. Total RNA (1 μ g) was reverse-transcribed as described above and diluted 10-fold. The integrity of the cDNAs was confirmed by RT-PCR using universal 18S rRNA gene primers (Wada & Satoh 1994). For each qRT-PCR assay, 1 μ l of cDNA was amplified in triplicate and in parallel with cDNAs to the synthetic RNA dilutions, which were used in each individual assay to generate a standard curve. The GAV copy numbers in experimental animals was calculated after real-time amplification, from the linear regression of that standard curve. The sensitivity of qRT-PCR compared to conventional endpoint 1 step and nested RT-PCR (Cowley et al. 2000) was verified by amplifying in parallel 119 gill, haemocyte, muscle, lymphoid organ and hepatopancreas tissue sample cDNAs by both techniques.

Statistical analysis. All numerical data were expressed as the mean \pm SE. Mixed-model analysis, Kaplan-Meier, log-rank chi square, Kruskal-Wallis and nonparametric Tukey-type multiple comparisons were used for statistical analyses (α = 0.05).

RESULTS

Sensitivity of qRT-PCR assay using serial dilutions of *in vitro* transcribed GAV RNA as a template

The analytical sensitivity of the qRT-PCR assay was determined with the serial dilutions of the GAV RNA standards ranging from 0.05 to 4.86 \times 10⁹ RNA tem-

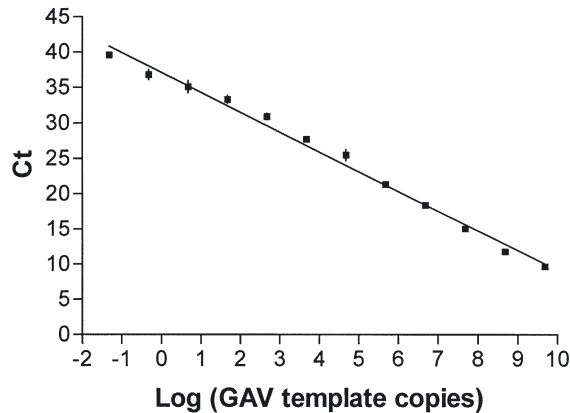


Fig. 1. Standard curve for gill-associated virus (GAV) copy number vs cycle threshold (Ct) value. Purified *in vitro* transcribed GAV RNA was serially diluted from 4.86×10^9 to 0.05 template copies per qRT-PCR reaction using GAV-free RNA as a diluent and as a template for the reverse transcription followed by the real-time RT-PCR. Resulting Ct values are plotted against the logarithm of their respective copy numbers

plate copies per qRT-PCR reaction. A linear relationship was observed between the Ct values and the log of the input RNA template copies with a regression coefficient (r^2) >0.98 (Fig. 1). The mean Ct values ranged from 9.66 ± 0.08 for 4.86×10^9 template copies to 39.59 ± 0.24 for 0.05 template copies (Table 1). Negative controls did not yield a detectable amplification product (Ct values of 40). Successful consistent amplifications were observed with as little as 0.5 viral template copies.

The same cDNAs were used to determine the sensitivity limit of the conventional GAV RT-nested PCR using 10 ng total RNA per reaction. The sensitivity of

first and second (nested) amplification steps were 486 and 0.5 GAV template copies ng^{-1} total RNA, respectively (Fig. 2). When 0.05 GAV template copies were used as a template, the amplicon was detected in 1 out of 3 samples tested (data not shown).

Specificity of real-time RT-PCR for GAV

The specificity of the real-time RT-PCR assay was determined through the amplification of lymphoid organ and muscle RNA isolated from both GAV chronically infected *Penaeus monodon* and GAV-free *Fenneropenaeus merguensis* juveniles. Chronically infected samples gave a positive signal during the real-time amplification, while no signal was observed in the GAV free samples. When the amplified products were run on a 2.5% agarose gel containing $0.5 \mu\text{g ml}^{-1}$ ethidium bromide, a single fragment of less than 100 bp was observed in the GAV-positive samples while no products were observed in the negative samples (data not shown).

Pathogenicity and GAV load in the viral inoculum

The pathogenicity of the inoculum was tested by injecting a group of 40 juvenile *Penaeus monodon*, all of which died by Day 12 post-injection, compared to 25% mortality in the control animals injected with sterile saline (data not shown). To relate this pathogenicity to the number of GAV RNA template copies injected, RNA extracted from the viral inoculum was tested by qRT-PCR and found to contain 8.6×10^4 GAV template copies ng^{-1} total RNA. From the linear regression of the RNA extraction efficiency assay (r^2 of 0.98), it was estimated that 200 μl of viral inoculum contained 19.08 μg RNA and thus ca. 1.6×10^9 GAV RNA template copies.

Prawn survival following treatment

Following experimental injection of 15 adult *Penaeus monodon* with 200 μl of viral inoculum, all prawns held across the 3 tanks developed lethargy and gross signs typical of GAV infection including red coloration of the appendages, tail fan and mouth parts (Spann et al. 2000). The 5 noninoculated prawns stressed by injection of saline solution alone developed similar gross signs of GAV infection but appeared more active.

Table 1. Cycle threshold (Ct) values of replicate assays with gill-associated virus (GAV) serial dilutions. SE = standard error

RNA standard GAV template copy no.	Ct values				Mean	SE
	A	B	C	D		
4.86×10^9	9.44	9.66	9.74	9.8	9.66	0.078
4.86×10^8	11.46	11.66	11.86	12.15	11.7825	0.147
4.86×10^7	14.91	15	15.06	15.19	15.04	0.058
4.86×10^6	18.14	19.08	18.36	18.05	18.4075	0.233
4.86×10^5	20.85	20.92	21.22	22.4	21.3475	0.359
4.86×10^4	24.06	24.25	26.75	26.94	25.5	0.778
4.86×10^3	26.97	27.08	28.28	28.61	27.735	0.416
486	30.05	30.28	31.63	31.75	30.9275	0.443
48.6	32.03	32.73	34.03	34.47	33.315	0.565
4.86	32.93	34.9	35.44	37.19	35.115	0.877
0.49	35.48	35.67	37.81	38.26	36.805	0.717
0.05	39.16	39.19	40	40	39.5875	0.238
NTC ^a	40	40	40	40	40	0

^aNo template control

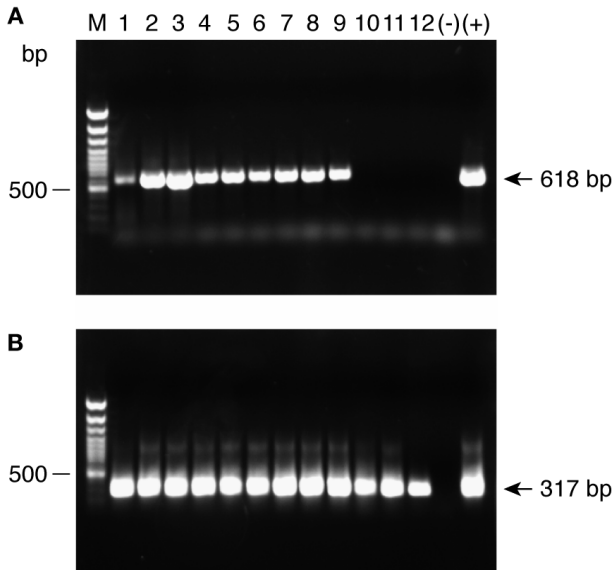


Fig. 2. RT-nPCR amplification of GAV RNA serial dilution. GAV template copies ng^{-1} total RNA. Lane 1: 4.86×10^9 , Lane 2: 4.86×10^8 , Lane 3: 4.86×10^7 , Lane 4: 4.86×10^6 , Lane 5: 4.86×10^5 , Lane 6: 4.86×10^4 , Lane 7: 4.86×10^3 , Lane 8: 486, Lane 9: 48.6, Lane 10: 4.86, Lane 11: 0.49, Lane 12: 0.05, (-) no template control, (+) positive control. (A) RT-PCR detection of 618 bp products and (B) nested PCR detection of 317 bp products. M = 100 bp DNA Ladder Plus (MBI Fermentas)

Cumulative mortalities reached 100% within 5 to 9 d in the inoculated treatment, and 40% on Day 5, after which mortalities stabilised in the non-inoculated group (Fig. 3). There was a significant difference in survival between the 2 treatments (log rank χ^2 5.264, $p = 0.02$).

Quantitative detection of GAV in experimental animals

For all experimental prawns, the GAV load in various tissues at different time points was determined by qRT-PCR. All tissues at all time points tested GAV positive by qRT-PCR. Following the acclimation period (Time 0), the mean viral load was $445 (\pm 317)$ GAV template copies ng^{-1} of total RNA in the haemocytes and $2.62 \times 10^5 (\pm 1.64 \times 10^5)$ GAV template copies ng^{-1} of total RNA in the spermatophores. The qRT-PCR data also showed that the distribution of GAV in tissues varied through time in each individual. Moreover, an increase in GAV load was observed in all experimentally inoculated as well as all non-inoculated individuals over the duration of the experiment (Fig. 4). Circulating haemocytes experienced a significant increase in GAV load regardless of the treatment (mixed-model analysis, $p = 0.010$) (Fig. 5). In the inoculated treat-

ment, this increase in GAV viral load reached a plateau 8 d post-infection, followed by a slight decrease in viral concentration until Day 9, the time at which the last individual died. The increase in viral concentration was significantly different between the 2 treatments (mixed-model analysis, $p = 0.047$).

The qRT-PCR also revealed that GAV was systemically distributed in all prawns tested, although significant differences in viral titre were detected among tissues in both treatments (Kruskal-Wallis χ^2 31.22, $p = 0.001$) (Fig. 6).

Diagnostic sensitivity of qRT-PCR compared to conventional RT-nested PCR

To estimate the diagnostic sensitivity of the qRT-PCR compared to conventional 2-step RT-nested PCR, prawn tissue RNA samples were processed in parallel by both techniques. The qRT-PCR and the RT-nested PCR had the same sensitivity and both tests consistently detected at least 1 GAV copy (0.22 GAV RNA template copies ng^{-1} of total RNA), which was the lowest viral concentration detected among the experimental tissues. Two out of 119 samples were negative by RT-nested PCR but positive results were obtained when the nested PCR was repeated. The lowest GAV load detected by endpoint analysis in the 1-step RT-PCR was 5.7 GAV template copies ng^{-1} of total RNA, while 4 samples ranging in viral concentration between 63 to 258 GAV template copies ng^{-1} total RNA were negative by first-step amplification. All these samples were positive in the nested amplification step and in the real-time RT-PCR assay.

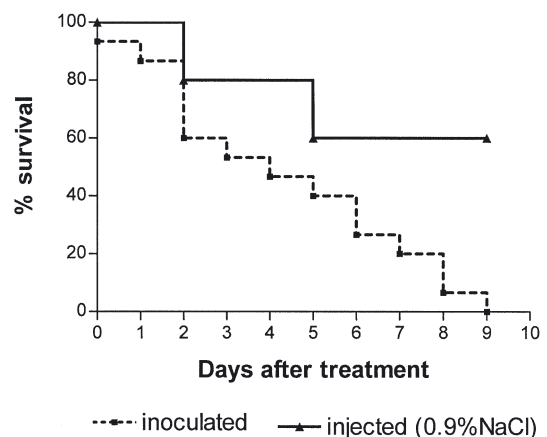


Fig. 3. *Penaeus monodon*. Survival curves following viral inoculation and repetitive bleeding. The stressed non-inoculated control group was injected with a 0.9% saline solution and subject to repetitive bleeding

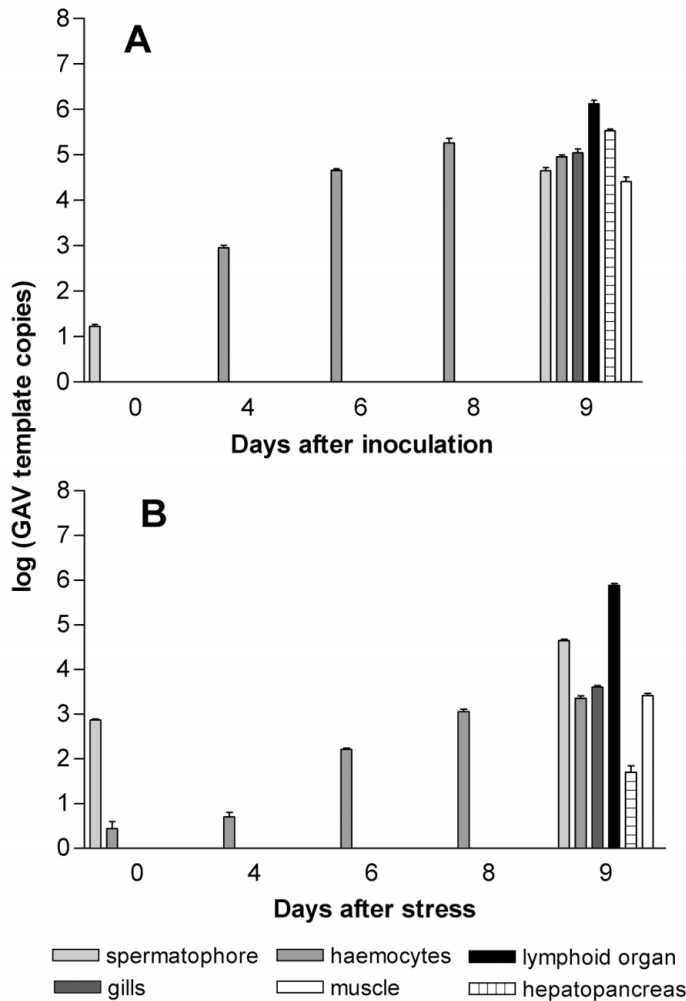


Fig. 4. *Penaeus monodon*. Time-course and tissue distribution of GAV infection in a single adult injected with (A) 200 μ l of viral inoculum or (B) 200 μ l of 0.9 % NaCl. Haemocyte samples were taken before and 4, 6, 8 and 9 d post-treatment. Spermatophore samples were taken before and 9 d after treatment. On Day 9, a moribund prawn in the inoculated treatment (A), and prawns exhibiting signs of stress such as lethargy and red coloration from the control treatment (B) were dissected and samples taken from the spermatophore, gills, lymphoid organ, muscle and hepatopancreas. RNA was extracted from these tissues and the GAV load was determined by real-time quantitative RT-PCR using 5 ng of cDNA (total RNA equivalents)

DISCUSSION

Recent surveys revealed that the prevalence of chronic GAV infection approaches 100% in wild and farmed *Penaeus monodon* along the east coast of Australia (Cowley et al. 2002a, Walker et al. 2001). Whereas conventional detection systems indicate whether GAV is present or absent in prawns, they only give a crude estimation of the abundance of GAV,

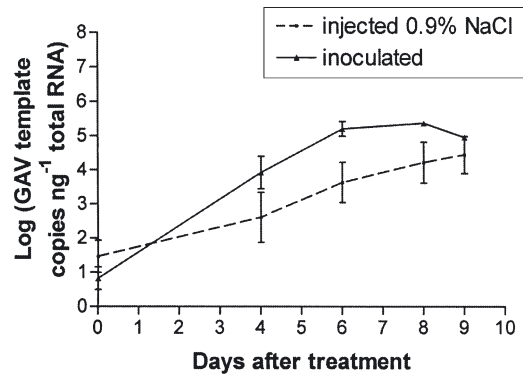


Fig. 5. *Penaeus monodon*. Time-course of GAV infection in the circulating haemocytes in a population of *P. monodon*. A group of 15 animals was injected with 200 μ l of viral inoculum (inoculated group). A placebo control group was injected with a sterile 0.9% NaCl solution. Individual prawns were bled before and on Days 4, 6, 8 and 9 following treatment. Values represent the population mean of surviving animals for each treatment at each time point. Bars represent \pm SE of the mean

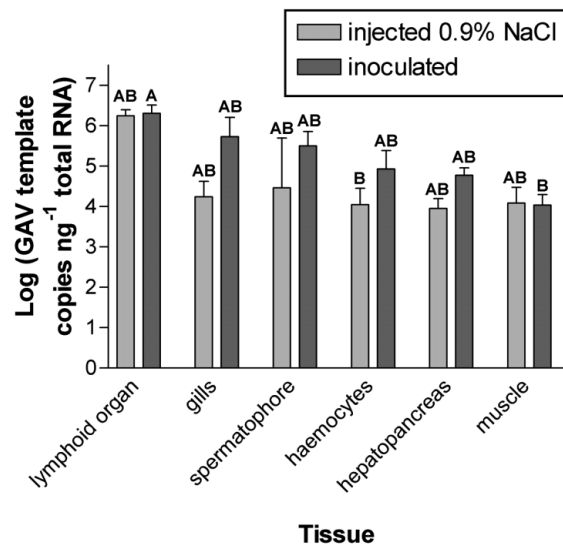


Fig. 6. *Penaeus monodon*. qRT-PCR detection of GAV levels in tissues dissected from moribund prawns throughout the experiment. Levels represent the mean (\pm SE) for the ‘super-infected’ and placebo control groups. Levels not connected by the same letter are significantly different. (Non-parametric Tukey-type multiple comparison test. $\alpha = 0.05$)

which provides farmers with little information on the health status of their cultured stocks. To manage and minimise the likelihood of disease outbreaks, there is a need for a method that enables the measurement of changes in viral concentration at individual and population levels. Historically, the determination of severity of GAV infection was delineated by the severity of gross signs, histopathology and the tissue distribution of the virus identified by *in situ* hybridisation or trans-

mission electron microscopy. However, such diagnostic methods can be subjective, are labour-intensive, and in most cases require culling of the animals. In contrast, the TaqMan qRT-PCR assay described here requires only small amounts of prawn tissue that can be collected non-sacrificially and is extremely rapid, specific and highly sensitive in detecting GAV. Moreover, the qRT-PCR could accurately quantify GAV RNA and thus infection levels and the Ct values displayed a linear relationship over a wide concentration range of input RNA from 0.5 to 4.86×10^9 template copies. Furthermore, by using dilutions of *in vitro* transcribed RNA instead of plasmid DNA to quantify GAV infection levels, the efficiency of the reverse transcription process was taken into account. Although qRT-PCR can accurately quantify the GAV RNA template number, it should be noted that this does not directly relate to the number of infectious virus particles. GAV dsRNA replicative intermediates have been detected in total RNA isolated from infected cells (Cowley et al. 2002b), and cDNA generated to both (+) and (-) sense RNA strands will be amplified in the PCR. Moreover, non-encapsidated, filamentous nucleocapsid precursors that contain GAV genomic RNA, but which are likely to be far less infectious than enveloped particles, are usually observed in far greater numbers in infected cells than are mature virions (Spann et al. 1997).

The sensitivity of the qRT-PCR is equivalent to that of the nested RT-PCR targeted to the same region of the GAV genome (Cowley et al. 2000) and could reproducibly detect down to 0.5 template copies of synthetic GAV RNA per qRT-PCR reaction. The limit of detection observed here is similar to the previously reported ~0.24 genome equivalents (Cowley et al. 2000). In this previous report, the authors utilised purified plasmid DNA instead of serially diluting *in vitro* RNA transcripts into virus-free RNA as template for the serial dilutions in the standard curve. When using prawn RNA as a template for endpoint-nested RT-PCR and real-time RT-PCR, both techniques were able to detect 1 template copy of GAV, which was the lowest viral concentration observed in these experimental animals. The 1st-step RT-PCR was able to detect 28 GAV template copies (5.7 GAV template copies ng^{-1} of total RNA) but did not detect GAV in some tissues in which up to 258 GAV template copies ng^{-1} RNA were estimated by qRT-PCR. This suggests that the detection limit of the 1-step RT-PCR resides somewhere within this range but, due to the 618 bp amplicon size, may be more affected by slight fluctuations in RNA integrity than the qRT-PCR (81 bp amplicon). Overall, the detection limit of qRT-PCR was only marginally superior to conventional RT-nested PCR for both synthetic and prawn RNA. Moreover, the diagnostic capability of both tests was comparable as the RT-nested PCR

failed to detect GAV in only 2 out of 119 *Penaeus monodon* tissue RNA samples, although these 2 samples were scored as GAV-positive in repeat nested PCR tests, that were all positive in the qRT-PCR.

The qRT-PCR detected GAV in all tissues tested from placebo and experimentally infected *Penaeus monodon*, although there were significant differences in viral loads between tissue types and between prawns at various stages of infection. This systemic distribution of virus is characteristic of an acute infection stage, and supports previous reports of GAV tissue distribution determined by *in situ* hybridisation (Tang et al. 2002, Spann et al. 2003). The high initial GAV levels found in this study for spermatophores supports previous observations with endpoint RT-PCR (Walker et al. 2001, Cowley et al. 2002a, Spann et al. 2003).

By non-lethal collection of spermatophore and haemocytes, the qRT-PCR was able to track changes in GAV infection levels over time in individual prawns. In chronically infected *Penaeus monodon* super-infected with GAV, the viral load in haemocytes increased rapidly ~ 10^5 -fold by Day 6 post-infection. At this time, 70% cumulative mortality had occurred and all prawns had died by Day 9, suggesting the mortality was directly attributable to the high GAV infection levels. Significant but lower increases in GAV loads of ~ 10^3 -fold were also seen in the haemocytes sequentially collected from placebo-injected, chronically infected *P. monodon*. It appears that the handling stress alone associated with haemolymph collection can induce an increase in GAV infection levels. Cumulative mortalities among this group reached 40% by Day 5 post-placebo injection, after which mortalities ceased to occur even though haemocyte levels of GAV continued to increase until the experiment was terminated on Day 9. Comparisons of mortality rates and qRT-PCR estimates of haemocyte infection levels in the control and super-infected prawns suggest that the rate of GAV replication, in addition to the overall viral load, may impact on whether a prawn succumbs to disease.

Over the 9 d of the experiment, a marked increase in the GAV infection levels initially detected in chronically infected *Penaeus monodon* collected from the wild occurred following injection of a placebo inoculum and subsequent regular bleeding to collect haemolymph. These prawns were used as controls for the group super-infected with GAV and the rapid increase in virus levels was unexpected. However, it clearly shows the stress of captivity, human handling and bleeding can increase overall virus levels. If haemolymph sampling alone induces viral replication, future experimental designs to evaluate the effect of stress on GAV replication will require a non-bled (unstressed) control. Indeed, the very act of shifting

prawns to new environments such as experimental tanks, may be sufficient to accentuate viral replication. If so, only subtle changes in the environment may be sufficient to induce disease outbreaks. Although sampling of individuals representative of treatment populations is an option, high initial natural variation in haemocyte viral loads of individual prawns was observed in the wild-caught broodstock used in the present study. Such variation in viral load between individuals will require a large sample size to obtain meaningful and statistically significant results. In addition, a true negative control (GAV-free prawns) would be desirable for these types of experiments. However, at present there are no certified GAV-free stocks available within Australia.

Mortalities have been associated with environmental stress at various phases of prawn production (Flegel & Pasharawipas 1998, Vidal et al. 2001). In *Penaeus monodon* hatcheries in eastern Australia, mortalities among wild-caught broodstock are known to commonly occur following eyestalk ablation, repeated handling and spawning (M. R. Hall et al. unpubl. data). Chronic GAV infection is endemic in the wild broodstock population that supplies these hatcheries (Cowley et al. 2000, Walker et al. 2001) in our evidence of high viral loads in some individuals, and that stress associated with handling and invasive clinical procedures can induce elevated viral loads and mortalities, offers an explanation for this problem. The TaqMan qRT-PCR assay for GAV described here is rapid, sensitive, quantitative and conducive to high-throughput diagnostic and epidemiological applications. Whereas this assay is impractical for routine use by the average farm or hatchery due to cost limitations, it will allow researchers to evaluate the effect of specific stressors or environmental triggers on GAV infection levels and the transition to acute infection and associated disease in *P. monodon* chronically infected with GAV. Such information will be valuable in the investigation of technological and management strategies to avoid increases in GAV levels in farmed *P. monodon* and thus of GAV-induced disease outbreaks.

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