

**The epidemiology of
gill-associated virus in *Penaeus
monodon* and the development
of alternative detection methods**

Thesis submitted by

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DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

James Munro

DECLARATION ON ETHICS

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the National Statement on Ethics Conduct in Research Involving Humans (1999), the Joint NHMRC/AVCC Statement and Guidelines on Research Practice (1997), the James Cook University Policy on Experimentation Ethics; Standard Practices and Guidelines (2001), and the James Cook University Statement and Guidelines on Research Practice (2001). The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review Committee (approval numbers A746, A832, A947).

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ABSTRACT

The hypothesis for this project was that gill-associated virus (GAV) affects production of *Penaeus monodon* on Australian farms. The main objectives of the research were, firstly; to determine if there is a relationship between the degree of GAV infection and the production of *P. monodon* from the examined farms. Secondly, to develop low cost detection methods for GAV and by inference, yellow head virus (YHV) which are applicable to the prawn farming industry in both Australia and developing countries.

The first objective was achieved by sampling a total of forty five prawn ponds from three prawn farms at both one month poststocking and at one week preharvest for the prevalence and semi-quantitative load of GAV using reverse transcription nested polymerase chain reaction (RT-nPCR) as the detection method. The three prawn farms were situated at different geographical positions along the east coast of Australia to reduce the possibility of location bias. The three locations were: 1) Palmers Island (northern New South Wales) - 29° 44' South, 153° 26' East; 2) Woongoolba (southern Queensland) - 27° 73' South, 153° 32' East and 3) Cardwell (northern Queensland) - 18° 27' South, 146° 02' East.

Prior to screening the three prawn farms for the affect of GAV, two criteria needed to be met. Firstly, to be able to determine if plasmid contamination had occurred within the PCR procedure, due to the high prevalence of GAV in *P. monodon* within Australia a high number of positives were expected and it would be difficult to detect false positives from plasmid contamination and secondly; to determine if the published RT-nPCR detection method for GAV could be used semi-quantitatively to compare levels of GAV infection between prawns. To fulfil these two criteria, firstly, a synthetic positive control for GAV RT- nPCR was developed. This PCR control produced larger amplified products in the outer and inner nest than the diagnostic test with the same primers. This technique is advantageous over traditional cloning of the diagnostic PCR product itself by making it visually easy to detect plasmid contamination and thus, prevent false positives.

To determine if RT-nPCR could be used to determine the semi-quantitative load, GAV from homogenised *P. monodon* gill tissue was purified on a continuous sucrose gradient. The RNA was then isolated from GAV and ten-fold serially diluted into crayfish (*Cherax quadricarinatus*) RNA that was free of GAV. These serial dilutions were then reverse transcribed into cDNA and amplified with PCR. It could be seen that as the original sample of GAV became increasingly diluted, the corresponding amplified product became progressively lighter when visualised on an agarose gel containing 0.5 µg/ml ethidium bromide under ultraviolet light. It was concluded that the RT-nPCR could be used as a semi-quantitative tool for the subsequent cohort study.

The ponds examined were categorised into four groups; i) GAV related outbreak – low production, ii) GAV related outbreak – emergency harvest, iii) no GAV related outbreak – low production, and iv) no GAV related outbreak – high production.

The study found that GAV had a strong association with reduced production from the farms. Ponds with GAV related outbreaks had a statistically higher initial prevalence (75.5 % – 80.7 %) and a higher increase in prevalence (16.3 % - 20.1 %) over the production period. While the ponds with no GAV outbreak – low production had the lowest initial prevalence (59.4 %) but the largest increase in prevalence (36.2 %) over the production period and the ponds with no GAV outbreak – high production had the lowest increase in prevalence (12.8 %) over the production period.

The association of GAV with reduced production was also seen with respect to the semi-quantitative loading of the infected prawns, as the ponds with GAV related outbreaks had the highest loading (2.6 – 2.9) (maximum being 4) and the highest percentage increase (41.8 % - 42.3 %) at harvest. While the ponds with no GAV outbreak – low production had a moderate increase (39 %) in GAV load and the ponds with no GAV outbreak – high production had the lowest increase (14 %) in load.

It was concluded that the prevalence and loading of GAV are strongly associated with the severity of disease; with a increase in GAV correlating with a decreased production. Ponds with a higher initial prevalence and higher increase in load of

GAV suffered GAV-related outbreaks. Ponds with low initial prevalence of GAV but with a high increase in prevalence and viral load over the production period suffered low level mortality resulting in no outbreak being identified, yet low production. The ponds that had moderate to low initial prevalence of GAV with a low increase in prevalence and load of GAV over the production period incurred no GAV related outbreak – high production.

The second objective was achieved through the development of two alternative detection methods for GAV. Firstly, polyclonal antibodies (PABs) from chickens and monoclonal antibodies (MAbs) derived from mice were produced against GAV and a capture ELISA was developed. Secondly, haemagglutination (HA) using chicken erythrocytes was used to detect GAV. These diagnostic tests for GAV were then tested for sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and overall accuracy when compared to the RT-nPCR as the gold standard for agreement with the ELISA or compared to the ELISA for comparison with HA.

PABs from chickens were produced against the 116 kDa and 64 kDa protein of GAV seen in Western blots. The development of the PABs was based on the report that YHV consisted of four structural proteins of approximately 170, 135, 67 and 20 kDa. However it was later reported that the 170 kDa protein may have originated from prawn cells. The PABs reacted to this 170 kDa. However, due to the specificity of the MAbs, this did not interfere with the developed capture ELISA. Of the 11 MAbs developed against the 20 kDa protein of GAV, all were IgM isotype. Monoclonal antibody 3K5-11 was used in immunohistochemical studies, Western blot analysis and affinity purification to demonstrate specificity to GAV.

Haemagglutination using chicken erythrocytes tested the haemolymph, gill, lymphoid organ, heart, subcutaneous tissue, eye stalk, pleopods, uropods and the central nerve cord for agglutination activity in 100 *P. monodon*, with the haemolymph and gill tissue giving the highest end-point titres of 1:1370 and 1:361, respectively. The sensitivity of HA was demonstrated by testing two different populations of *P. monodon*, which had a highly significant difference ($F = 56.4$, $DF = 4, 88$, $P < 0.001$) in HA activity, indicating a difference in viral load. By testing

three other penaeid species ($n = 20$ each), *Penaeus esculentus*, *Penaeus merguensis* and *Penaeus longistylis*, and the crayfish, *C. quadricarinatus*, it was demonstrated that natural agglutinins were not causing the high agglutination in the population of *P. monodon*. There was no effect of freezing and thawing of samples on HA activity. The speed and low cost of HA makes it a very useful tool, particularly in the developing world, for on-farm testing of penaeid prawns to indicate YHV and GAV loads which can contribute to management practices with respect to the harvesting of ponds.

The two developed tests were compared for agreement using 120 *P. monodon* for the presence of GAV. Initially, the ELISA was compared to the RT-nPCR and then the HA was compared to the ELISA. For the ELISA, the sensitivity was 97 %, the specificity was 65 %, the PPV was 93.3 %, the NPV was 81.3 % and the overall accuracy was 91.7% when using an optical density of greater than 0.75 as a positive result. The HA had a sensitivity of 88 %, specificity of 75 %, PPV of 79%, NPV of 61 % and an overall accuracy of 73 % compared to the ELISA or an estimated accuracy of 66.9 % when compared to the RT-nPCR when using an HA titre of greater than 16 as a positive result.

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LIST OF ABBREVIATIONS

A ₂₆₀	absorbance at 260 nm
A ₂₈₀	absorbance at 280 nm
ABTS	2,2'-azino-di-(3-ethylbenzthiazoline-6-sulphonic acid)
APS	ammonium persulphate
AU	Australian
BDS	bovine donor serum
bp	base pairs
BSA	bovine serum albumin
cDNA	complimentary deoxyribonucleic acid
CO ₂	Carbon dioxide
d	days
DAB	3, 3'-diaminobenzidine tetrahydrochloride
D.P.X	di-n-butylphthalate polystyrene xylene
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
FAO	food and agriculture organisation of the United Nations
FBS	foetal bovine serum
g	gram
<i>g</i>	gravity
GAV	gill-associated virus
h	hours
HA	haemagglutination
HAT	hypoxanthine-aminopterin-thymidine
H&E	haematoxylin and eosin
HRP	horseradish peroxidase
HT	hypoxanthine-thymidine
ICTV	International Committee on Taxonomy of Viruses
IHHNV	infectious hypodermal and haematopoietic necrosis virus
kb	kilobase

kDa	kiloDalton
LOV	lymphoid organ virus
LO	lymphoid organ
LPV	lymphoid parvo-like virus
LSD	least significant difference
MAbs	monoclonal antibodies
MAb	monoclonal antibody
MBV	monodon baculovirus
MCMS	mid-crop mortality syndrome
MoV	Mourilyan virus
MW	molecular weight
NDV	newcastle disease virus
NPV	negative predictive value
O.D	optical density
OPI	oxaloacetate-pyruvate-insulin media supplement
ORF	open reading frame
PABs	polyclonal antibodies
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PL	postlarvae
PPV	positive predictive value
PVDF	polyvinylidene fluoride
RBC	red blood cell
RNA	ribonucleic acid
RT-nPCR	reverse transcription nested polymerase chain reaction
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium-dodecyl-sulphate
SDS-PAGE	sodium-dodecyl-sulphate poly-acrylamide gel electrophoresis
SEM	scanning electron microscopy
SEMBV	systemic ectodermal and mesodermal baculovirus
SMV	spawner-isolated mortality virus
SPSS	statistical package of social sciences
TAE	tris/acetate/EDTA

TCID ₅₀	50% tissue culture infectious dose assay
TE	tetracycline
TEM	transmission electron microscope
TEMED	tetramethylethylenediamine
TNE	tris/NaCl/EDTA
U	units
US	United States
WSSV	white spot syndrome virus
YHLV	yellow head-like virus
YHV	yellow head virus
v	volts

CHAPTER 1

General Introduction

Penaeid aquaculture was originally performed as “catch and hold” culture systems. For centuries, Southeast Asian farms have been producing incidental crops of wild prawns in tidal ponds. With the advent of ever advancing technology and the increasing requirement for low cost protein as a food source, the penaeid prawn culture has advanced from its experimental beginnings into major industries providing hundreds of thousands of jobs, billions of dollars in revenue, and an expansion of the world’s food supply with a high value crop (Lightner and Redman 1998). The importance of disease within the industry has increased proportionally with the growth of the prawn industry.

Until the early 1990’s, prawn aquaculture exhibited astonishing growth of 16.8 % per annum between 1984 and 1995 (Subasinghe, Bartley, McGladdery, and Barg 1998). Recently, however, diseases have had devastating impacts on prawn farming. An economic impact assessment from Lundin (1997) reported that in 1994, just over two billion United States (US) dollars were lost due to disease. Disease outbreaks have continued to cause major losses.

Diseases with viral aetiologies have been the most important cause of economic loss in the majority of countries (Fegan and Clifford 2001). The emergence of ‘new’ viruses is rapidly increasing. For example, in 1990, Lightner, Bell, and Redman reported six viruses affecting penaeid prawns. By 1992, the list of known viruses affecting penaeid prawns had increased to 12 (Lightner 1996) and presently, approximately 20 viruses have been reported in penaeid prawns. Three of these viruses have been responsible for the most severe losses. These are, in order of their discovery, Taura syndrome virus (TSV), yellow head virus (YHV) and white spot syndrome virus (WSSV).

Australia’s zoogeographical isolation from Southeast Asia enabled it to be fairly late with respect to the major viral disease outbreaks. However by 1994, major disease outbreaks were occurring within the Australian prawn farming industry, with

mortalities reaching as high as 80% (Owens, Haqshenas, McElnea, and Coelen 1998). This disease outbreak was dubbed mid-crop mortality syndrome (MCMS). There were four viruses associated with this syndrome. Initially it was thought to consist of only two viruses, spawner-isolated mortality virus (SMV) and gill-associated virus (GAV). However, recently it was reported that infectious hypodermal and haematopoietic necrosis virus (IHHNV) (Krabsetsve, Cullen, and Owens 2004) which is similar to SMV with respect to being a non-enveloped DNA parvo-like virus, and Mourilyan virus (MoV) (Cowley, McCulloch, Spann, Cadogan, and Walker 2005) which is similar to GAV with respect to being a enveloped RNA virus, were also present during the outbreaks. These viruses could have equally influenced the mortality of the infected prawns as they have all been associated with disease.

There were two main objectives of this study. Firstly, to determine if GAV and by inference, YHV have an association with disease in *Penaeus monodon* within the prawn farming industry.

GAV and YHV are reported to be geographic topotypes, sharing 85.1% sequence identity (Cowley *et al.*, 1999) and have been reported to be highly pathogenic to *P. monodon*. The pathogenicity of GAV was determined by inoculation of filtered homogenates of lymphoid organ, gills and whole cephalothoraces from *P. monodon* that were positive for GAV, resulting in mortality to other *P. monodon* which were already infected with GAV (Spann, Cowley, Walker, and Lester 1997a; Vega, Degnan, Hall, Cowley, and Wilson 2004). The method of determining the pathogenicity of a virus via crude inoculations should be obsolete due to our current knowledge of multiple infections of viruses within penaeid prawns. Determining pathogenesis with this method is only accurate when either pathogen-free stock or crustacean cell lines are available with viable pure virus.

At present, there are approximately twenty research papers describing different aspects of GAV and only one of those papers by Callinan, Jiang, Smith, and Soowannayan (2003a) attempts to determine if GAV is associated with causing an economic impact on Australian prawn farms.

In the present study, the first objective investigates the association of GAV with survival on three prawn farms located over three different geographic locations along the eastern coast of Australia. This aims to determine whether GAV is associated with disease and mortality within the Australian *P. monodon* aquaculture industry. The second objective was to develop detection methods for GAV, and by inference YHV, which are cost effective for small scale prawn farmers in Australia and developing countries. There are currently many molecular techniques for the detection of GAV/YHV. Despite these PCR techniques being highly sensitive for the detection of the viruses, there are practical limitations to their widespread application. These limitations include the requirement for specialised equipment, expensive molecular reagents and well-trained personnel. Consequently, these high costs result in the assay being non-viable for the majority of prawn farmers or small support laboratories that screen for viruses. The following study aimed at developing non-molecular detection methods which were both sensitive and specific whilst being relatively low cost.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Properties of the Okavirus

The purpose of this literature review is to focus on the yellow head-like viruses. There has been considerable confusion with respect to the taxonomic classification of these viruses. Initially, the yellow head-like viruses were proposed to be a baculovirus due to their size and enveloped rod-shaped appearance (Chantanachookin, Boonyaratpalin, Kasornchandra, Direkbusarakom, Ekpanithanpong, Supamataya, Sriurairatana, and Flegel 1993). However, upon the discovery that the genome consisted of ssRNA, it was suggested that the virus was either a rhabdovirus or a coronavirus (Wongteerasupaya, Sriurairatana, Vickers, Akrajamorn, Boonsaeng, Panyim, Tassanakajon, Withyachumnarnkul, and Flegel 1995a). Loh, Tapay, Lu, and Nadia (1997) reported the genome to possibly be negative in polarity resulting in the virus being classified as *Rhabdoviridae*. Tang and Lightner (1999) subsequently reported that yellow head virus (YHV) was a plus-strand RNA virus via *in situ* hybridisation and sequence analysis. These latest results placed YHV into the corona-like viruses. Cowley, Dimmock, Spann, and Walker (2000a) reported that the yellow head-like virus genomes contain a ORF1a polyprotein containing a 3C-like Cys protease, an ORF1b coding sequence with replicase functions including an SDD polyprotein, and a helicase domain, an efficient – 1 ribosomal frameshift site at the ORF1a/1b overlap that will facilitate translation of a 759 kDa ORF1ab polyprotein. From this they concluded that the yellow head-like viruses were a unique member of the *Nidovirales*. The yellow head-like viruses have subsequently been placed as members of a new genus *Okavirus* of a new family *Roniviridae*, within the order *Nidovirales* (Mayo 2002). There are two other families within the order *Nidovirales*. These are *Coronaviridae* and *Arteriviridae* (Figure 2.1).

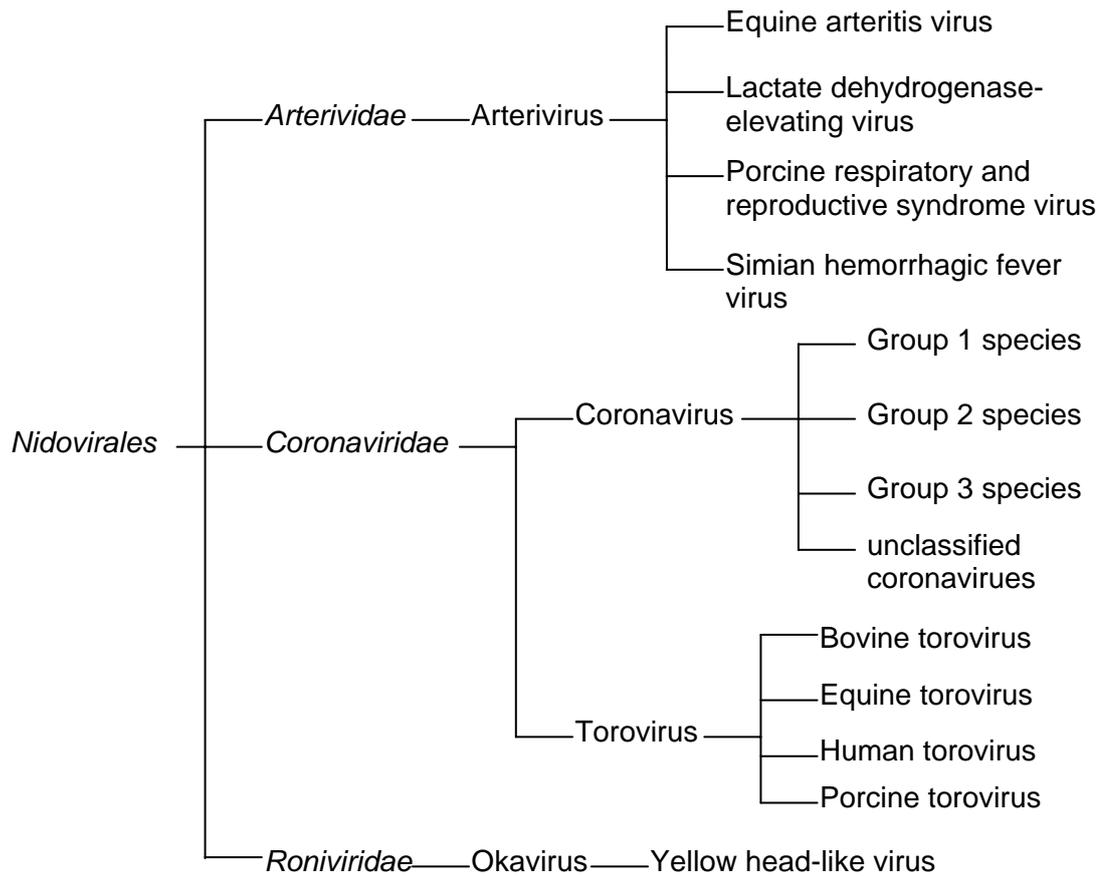


Figure 2.1. Family tree of the genera and families within the order *Nidovirales*

2.2 Viruses of the yellow head complex

Currently, the literature isolates the yellow head-like viruses into two distinct viruses, being gill-associated virus (GAV) and YHV. GAV is the junior synonym of lymphoid organ virus (LOV) which was reported in 1995 by Spann, Vickers, and Lester. LOV was reported to be found only in the lymphoid organ and was similar to YHV with respect to ultrastructural and cytopathological features. However, LOV had no association with disease and mortality (Spann *et al.*, 1995; Spann, Cowley, Walker, and Lester 1997a). GAV was subsequently reported as a pathogenic relative of LOV, found both in the lymphoid organ and the gills of infected *Penaeus monodon* (Spann *et al.* 1997a). With the development of a reverse transcription nested polymerase chain reaction (RT-nPCR) towards GAV it was reported that LOV had a 98.9% nucleotide identity to the GAV sequence, indicating that they are the same virus (Cowley, Dimmock, Spann, and Walker 2000b). However, the

method to determine the level of nucleotide similarity used only two clones to determine the 1.1 % (3/274) nucleotide variation.

In contrast, GAV had an 85.1 % nucleotide identity to YHV from a 577 bp region and a 83 % nucleotide identity to YHV from a 135 bp sequence of a cDNA clone. From this, YHV was reported to be a closely related geographic topotype of GAV. However, this study only performed sequences analysis on three YHV clones (Cowley, Dimmock, Wongteerasupaya, Boonsaeng, Panyim, and Walker 1999). Even though GAV and YHV are currently classified as distinct viruses, it appears that the research applied to form these conclusions was very limited. Due to the extremely small number of clones that were sequenced and the small sequence region, the research did not take into account the possibility of the thousands of mutants within the clones which can constitute so-called quasi-species within a population (van Regenmortel 2000) not to mention the possible natural genomic variation within the so-called two distinct viruses. Therefore, due to the small sample size, the sequence variation was not representative of the actual population and the actual nucleotide variation could be a lot larger or smaller than the reported variation resulting in either the viruses being the same virus or distinctly different. Since 1991, the International Committee on Taxonomy of Viruses (ICTV) has accepted the definition that “a virus species is a polythetic class of viruses that can constitute a replicating lineage and occupy a particular ecological niche”. van Regenmortel (2000) lists the following characteristics for discriminating between virus species:

- Relatedness of genome sequence
- Natural host range
- Cell and tissue tropism
- Pathogenicity and cytopathology
- Mode of transmission
- Physicochemical properties of virions
- Antigenic properties of viral proteins

Due to this, and the fact that the viruses are morphologically indistinguishable and cause the same gross disease, in this literature review, GAV and YHV will be referred to as the same virus, being ‘yellow head-like virus’ (YHLV).

2.3 Infection and morphology of yellow head-like viruses

A study by Lu, Tapay, Loh, Brock, and Gose (1995) reported that YHLV particles were detected in the gill, lymphoid organ, head soft tissue, heart, midgut, hepatopancreas, abdominal muscle, eyestalk and nerve cord of an experimentally infected *Penaeus vannamei*. Lu *et al.* (1995) reported that the lymphoid organ, gill and head muscle had a 50 % tissue culture infectious dose assay (TCID₅₀) titer (ml⁻¹) of 10⁶, while the midgut, abdominal muscle and heart had a TCID₅₀ titer (ml⁻¹) of 10⁵ and the nerve cord, hepatopancreas and the eyestalk had a TCID₅₀ titer (ml⁻¹) of 10⁴. These findings suggest that the lymphoid organ, gill and head muscle contained the highest number of infectious virions compared to the other tested tissue/organs. These results also indicated that the viral infection was systemic. Virions of similar morphological appearance to YHLV have also been reported in the optic nerve fibres and in the nerve cord of *P. monodon* (Smith 2000; Callinan *et al.*, 2003a). To confirm the entire viral distribution in prawn tissues, a more comprehensive examination needs to be conducted in other organs and tissues such as haematopoietic tissue, Y-organ, stomach, antennal gland, periopods, pleopods and uropods.

YHLV replication occurs in the cell cytoplasm, primarily in the prawn lymphoid organ, gills, haemocytes and connective tissues (Cowley, Dimmock, Spann, and Walker 2001). The YHLV virions are rod-shaped, enveloped particles containing helical nucleocapsids that mature by the process of budding through intracytoplasmic membranes (Chantanachookin *et al.* 1993, Spann *et al.* 1997a). The nucleocapsids exhibit striations with a periodicity of approximately 7 nm and are often observed in association with the distended endoplasmic reticulum (Spann *et al.* 1997a).

The virions vary between 160-186 nm by 38-50 nm and 183-200 nm by 34-42 nm in size and are often packed densely into vesicles, resembling paracrystalline arrays (Figure 2.2). Free virions are also observed in intercellular spaces probably via release from disintegrating cells (Chantanachookin *et al.* 1993; Spann and Lester 1997c). Within all stages of YHLV infected cells, virogenic stroma and filamentous YHLV nucleocapsids, 116-435 nm by 16-18 nm in size are often observed scattered

randomly within the cytoplasm (Spann and Lester 1997c). The nucleocapsid of YHLV becomes enveloped by passage through the endoplasmic reticulum or the virions have occasionally been observed invading the interstitial spaces of the lymphoid organ and gain their envelope by passage through the plasma membrane (Spann and Lester 1997c).



Figure 2.2. A YHLV infected *Penaeus monodon* lymphoid organ cell showing paracrystalline arrays of enveloped virions (A). Scales bar = 200 nm (Spann *et al.* 1995)

YHLV consists of 26,235 nucleotides that are organised into four open reading frames (Figure 2.3) (Cowley and Walker 2002). Initially YHLV was thought to consist of four structural proteins with the following estimated molecular weights: 170, 135, 67, and 22 kDa (Nadala, Tapay, and Loh 1997). The proteins were thought to represent, respectively the L (RNA transcriptase), G (spike), N (nucleocapsid), and M (matrix). However, Wang and Chang (2000) reported only three major YHLV proteins, being: 110, 63, and 20 kDa in size and suggested that the larger protein (170 kDa) reported by Nadala *et al.* (1997) was cellular in origin. However, it is possible that due to the techniques used to purify the virus by Wang and Chang (2000), the 170 kDa polyprotein may have been cleaved to produce the reported 110 and 63 kDa proteins.

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Figure 2.3. Organisation of the 26235 nt (+) ssRNA YHLV genome indicating translation features and deduced open reading frame (ORF) functions (Cowley and Walker 2002).

Haemagglutination (HA) activity from YHLV has been reported by Nadala *et al.* (1997). They reported that purified YHLV agglutinated chicken erythrocytes yielding a HA end-point titre of 1:256 and the virus was not eluted after 24 hours, suggesting the reaction was stable and that the virus lacked receptor-destroying enzymes. HA activity from YHLV infected prawns was confirmed by Munro and Owens (2005) while YHLV-free prawns demonstrated negligible HA activity. The protein responsible for the HA is thought to be similar to the HA protein on the outside of some Coronaviruses (Figure 2.4)

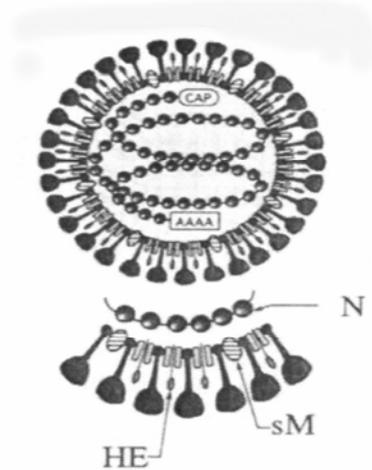


Figure 2.4. Diagrammatic representation of the structural components and schematic morphology of coronavirus virions. Note: N, nucleocapsid protein; sM, small-membrane protein; HE, haemagglutinin-esterase protein.

2.4 Disease and distribution of yellow head-like viruses

The YHLV throughout most of Southeast Asia is reported to be a highly pathogenic agent for cultured *P. monodon*, causing significant mortalities and adversely affecting the mariculture prawns in Thailand (Chantanachookin *et al.* 1993). The YHLV was first described in Thailand in 1990 by Limsuwan (Chantanachookin *et al.* 1993). Limsuwan named the new syndrome based on the light yellow colouration of the dorsal cephalothorax area and the general pale appearance of the infected prawn. The yellow appearance was a result of the underlying enlarged yellow hepatopancreas. Since this time, the disease has been associated with epizootic mortalities in Thailand. YHLV is reported to be one of the most highly virulent viruses of the causative agents in Thailand, since it is associated with massive mortality of *P. monodon*, the principle penaeid species cultured in Thailand (Sithigorngul, Chauychwong, Sithigorngul, Longyant, Chaivisuthangkura, and Menasveta 2000). Since the identification of the causative agent as YHLV in Thailand in 1990 and the resulting epizootic mortalities it was associated with, the virus has been associated with mortalities in penaeid prawns in Taiwan, Indonesia, Malaysia, China, Philippines, India and the Americas (Lightner 1996; Mohan 1996).

There has been only one reported occurrence of YHLV in the Americas at a *Penaeus setiferus* farm in Texas. The farm was in close proximity to a prawn processing plant and it was suggested that the virus was imported from Asia (Lightner, Redman, Poulos, Nunan, Mari, and Hasson 1997). The YHLV has also been detected in frozen prawns that had been imported into the US from Asia. At present, there is no evidence to show that YHLV is now present in wild or farmed prawns in the Americas.

Initially, YHLV principally infected pond-reared juvenile to sub-adult prawns of 5 to 15 g in size, especially at 50 - 70 days of grow-out (Lightner 1996). Since the initial epizootic, it has been suggested that the disease is now less severe than when YHLV was first isolated in Thailand. YHLV infection is now common in healthy prawns. This disease resistance is proposed to be from “active accommodation” by a tolerance mechanism involving binding of viral antigens to cellular receptors during the early life stages of the prawn (Flegel and Pasharawipas 1998).

This theory of “active accommodation” is supported in the literature. For example, transmission electron microscopy (TEM) of broodstock collected in Thailand prior to the initial reports of the virus, indicated that YHLV was present in one out of seven healthy broodstock sampled. During the peak of the YHLV epidemic in Thailand, YHLV was detected by TEM in gill samples from at least one prawn from 15 ponds with gross signs and a pond history that indicated YHLV was present. In the three ponds with no signs of YHLV disease, six out of six prawns sampled negative for YHLV by TEM. However, later in the YHLV epidemic, YHLV could be detected by TEM in gill samples from 33 out of 44 healthy prawns sampled from 11 ponds without signs of YHV (Walker, Cowley, Spann, Hodgson, Hall and Withyachumnarnkul 2001).

A study of 19 *P. monodon* broodstock collected from hatcheries in Thailand was conducted using RT-PCR on total nucleic acid extracted from gill tissue (Wongteerasupaya, Tongchuea, Boonsaeng, Panyim, Tassanakajon, Withyachumnarnkul, and Flegel 1997). All 19 prawns tested negative for YHLV. However, using a 2-step RT-PCR on the same prawns resulted in 15 of the 19 prawns (78.9%) testing positive for YHLV (Wongteerasupaya *et al* 1997). A survey

conducted in the Philippines on 219 healthy prawns with Western blot analysis also indicated a relatively high prevalence (24.2%) of YHLV infection (Natividad, Magbanua, Migo, Alfafara, Albaladejo, Nadala, Loh, and Tapay. 1999). The prevalence varied between 0 – 66.7% depending upon which districts were sampled. There was also evidence of a higher prevalence of infection in postlarvae (54.5%) than in broodstock (16.9%). With respect to the limited sensitivity of Western blot methods compared to 2-step PCR, the true level of chronic YHLV infection in the Philippines may be much higher. Yang, Shariff, Lee, Hassan (2000) also reported that YHLV occurs in high prevalence in Malaysia but it does not appear to have been associated with significant mortalities.

These studies suggest that a high proportion of apparently healthy *P. monodon* broodstock from some areas of Asia carry chronic infections of YHLV. It is highly likely that the previous TEM studies of broodstock and farmed prawns in Thailand would not have detected this low level of infection that is often only evident in 2-step PCR. Therefore, chronic YHV infection may have been highly prevalent in healthy prawns prior to the appearance of the disease. The increase in prevalence of infection detected by TEM could have been due to a general increase in viral load in the farmed prawns. The relationship between viral load and susceptibility to disease is the subject of ongoing research.

Within Australia, YHLV has been associated with significant mortalities which have adversely affected the prawn farm industry in Australia since at least 1996 (Spann, Donaldson, Cowley, and Walker 2000). YHLV infections can be chronic or acute. A chronically infected *P. monodon* with YHLV displays no gross signs of disease or tissue necrosis, while acute infections result in necrosis, disease and mortalities. Under experimental conditions YHLV is reportedly highly pathogenic, causing mortalities from 4 to 5 days after infection (Walker *et al.* 2001). In the farming environment, mortalities from YHLV are generally observed in prawns between 8 and 15 g, although prawns up to 40 g have exhibited signs of disease (Spann and Lester 1997).

The reported prevalence of YHLV within Australia in *P. monodon* broodstock from a sample size of 148 prawns captured in north eastern Queensland was 97.3%.

Prevalence of YHLV in postlarvae from a sample size of 50 was 100% and prevalence of YHLV in juveniles from a sample size of 56 was 98.2% (Walker *et al.* 2001). However, it was not reported how these samples were obtained, so it is unknown if they are from the same hatchery and/or the same broodstock.

Even with this high prevalence of YHLV, not all YHLV infected *P. monodon* express disease. Currently, the factors involved for YHLV-related disease to be expressed are poorly understood. Some hypotheses of these factors are; the viral load of parental broodstock, the initial viral load of postlarvae or an unknown environmental factor acting as a stressing agent.

YHLV has been reported to be highly pathogenic to *P. monodon* within Australia. The pathogenicity of YHLV was determined by inoculation of filtered homogenates of lymphoid organ, gills and whole cephalothoraces from *P. monodon* that were positive to YHLV, resulting in mortality from 7 to 8 days (d) post-inoculation (Spann *et al.*, 1997a). However, at the time of the pathogenicity trial there were at least five other viruses infecting Australian *P. monodon*. These viruses consisted of monodon baculovirus (MBV) (Doubrovsky, Paynter, Sambhi, Atherton, and Lester 1988), lymphoid parvo-like virus (LPV) (Owens, De Beer, and Smith 1991), IHNV (Owens, Anderson, Kenway, Trott, and Benzie 1992), spawner-isolated mortality virus (SMV) (Fraser and Owens 1996) and Mourilyan virus (MoV) (Cowley, *et al.*, 2005) which all could have possibly influenced the mortality of the *P. monodon* in this trial. This infection trial was repeated in 2004 by Vega *et al.* In that study, the prawns were again inoculated with filtered prawn homogenate containing levels of YHLV (not purified virus). They reported that 100% (15/15) of the YHLV injected prawns died compared to 40 % (2/5) of the controls while there was a significant increase ($P = 0.010$) in YHLV for both the infected prawns and the controls during the trial. However, the YHLV increase was significantly higher ($P = 0.047$) in the YHLV injected prawns than the control prawns. Again, no other viruses were tested for in that study or referred to as potential pathogens.

There are many papers that report YHLV to be pathogenic. However there is no substantial evidence to support this. Currently there is only an association with disease. Many papers report that severe necrosis of the lymphoid organ (LO) is a

typical lesion caused by YHLV (Boonyaratpalin *et al.*, 1993; Chantanachookin *et al.*, 1993; Lu, Tapay, Brock, and Loh 1994; Wang, Tang, Kou, and Chen 1996).

However, penaeid prawns with severe white spot syndrome virus (WSSV) exhibit the same marked LO necrosis (Pantoja and Lightner 2003). The current method of injecting YHLV infected crude homogenate of prawn tissue to determine pathogenicity should be obsolete with our current knowledge of possible multiple viral infections within the same prawn or homogenate sample. To demonstrate a direct pathogenic effect of YHLV, either pathogen-free stock would be required or a viable penaeid cell line would be needed with purified viable YHLV. Until these requirements are available, YHLV can only be said to be associated with disease.

2.5 Mode of infection of yellow head-like viruses

The modes of infection of the yellow head-like viruses have been placed into two general groups, horizontal transmission and vertical transmission (Figure 2.5).

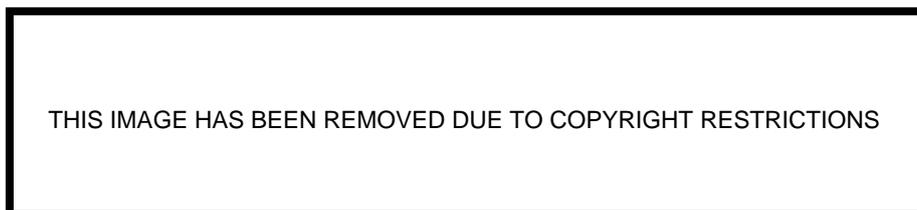


Figure 2.5. Model for the infection and disease cycle of yellow head complex viruses (Walker *et al.* 2001)

Horizontal transmission can occur when YHLV-free *P. monodon* either feed on infected carcasses, experience bath exposure to membrane-filtered tissue extracts, by cohabitation with infected prawns, or by direct experimental injection of the viral

inoculum (Walker *et al.* 2001). An experiment to determine the susceptibility of postlarval (PL) *P. monodon* of YHLV by ingestion showed that PL₂₀ died 7-10 days post-infection but PL₁₅ survived the exposure (Spann, Donaldson, McCulloch, Cowley, and Walker submitted). The available data suggested that disease was associated with viral loading. Walker *et al.* (2001) reported that YHLV from a diseased *P. monodon* caused mortalities after ingestion or immersion exposure but extracts from YHLV-infected healthy *P. monodon* caused infection without mortality. However, after the viral concentration of the healthy prawns had an equivalent titre to the diseased prawn, YHLV extracts from chronically infected, healthy *P. monodon* also induced disease, indicating disease is associated with viral loading.

The potential for vertical transmission was first reported by Chantanachookin *et al.* (1993). They reported that YHLV infection in larval offspring could occur from latent, asymptomatic infected, broodstock prawns. This theory was originally dismissed for YHLV because TEM screening of *P. monodon* in Thailand suggested that the prevalence of YHLV was low, and therefore, that vertical transmission was unlikely to contribute significantly to the occurrence of infection and disease on farms (Flegel, Boonyaratpalin, and Withyachumnarnkul. 1997). With more recent screening of broodstock with RT-nPCR, Walker *et al.* (2001) suggested that the prevalence of YHLV infection in Thailand may be significantly higher than originally indicated using TEM.

To determine if vertical transmission of YHLV contributes to the high prevalence of chronic infections in wild and farmed *P. monodon* in eastern Australia, Cowley, Hall, Cadogan, Spann, and Walker (2002) tested gonads and lymphoid organs for signs of YHLV from healthy male and female *P. monodon* broodstock and in fertilized eggs and from nauplii spawned from wild-fertilized females using RT- nPCR. They reported that the level of YHLV in wild *P. monodon* was generally low. However, high levels of YHLV were detected in moribund male broodstock reared in captivity for more than 12 months. The RT-nPCR product from spermatophore in these prawns were also significantly greater than those amplified from the lymphoid organ, which had previously been identified as the primary site of YHLV replication in chronically infected *P. monodon* (Spann *et al.* 1995). It was also reported that in 1 of

3 spermatophores examined by TEM, mature YHLV virions were detected in the seminal fluid but not in the sperm cells. The RT- nPCR for YHLV in eggs were positive, however, nauplii and protozoa were generally negative. This suggested that YHLV is associated with the egg surface and the majority of the virus is lost when the nauplii hatch and that the infection levels in the protozoa remain low. Cowley *et al.* (2002) suggested that this could be due to the lack of development of the lymphoid organ in larval and early postlarval life stages and is likely to limit potential infection levels. Walker *et al.* (2001) reported that RT- nPCR has detected YHLV in PL₅ to PL₁₅ both from hatcheries and experimental spawnings of *P. monodon*. Walker *et al.* (2001) suggested that viral replication in postlarvae is occurring at sufficient levels to be detected. Cowley *et al.* (2002) reported that the identification of lymphoid organ spheroid bodies and YHLV particles in ~1.2 g juvenile *P. monodon* grown from hatchery stocks (PL₆ and PL₂₀) suggests that at least some of the postlarvae were infected with YHLV. However, since individual postlarvae were not grown in isolation, it cannot be discounted that some juvenile infections may have occurred during the course of the grow-out through cannibalism or water-borne transmission. This horizontal transmission could promote translocation of YHLV and the potential infection of wild *P. monodon* in the vicinity of farms via water or through the escape of infected farmed prawns. The authors concluded that the high prevalence of chronic YHLV infection in *P. monodon* broodstock from northeastern Queensland and farmed prawns produced from these broodstock strongly suggests that this is perpetuated primarily by vertical transmission both in the wild and in hatcheries. However, in that paper, the authors did not comment on the likely survival of the YHLV infected progeny. They only tested eggs and nauplii. It is only an assumption that these postlarvae survive through to adulthood.

2.6 Interactions with other viruses

At present there are approximately 18 viruses that have been reported in penaeids. Not all of these viruses have been shown to cause disease or to interact with each other once they have infected the prawn. Within the YHLV, there are two main groups of viruses that have been indicated to cause disease when a dual infection

occurs. The two groups of viruses that interact with each other are the YHLV with WSSV and YHLV with SMV.

WSSV was previously classed as a baculo-like virus (Nadala, Tapay, and Loh 1998). However, it has recently been placed into a new family and genus, being family *Nimaviridae*, genus *Whispovirus* (Mayo 2002). It was first seen as a dual infection with YHLV in Thailand in *P. monodon* in 1993 and was originally named Systemic Ectodermal and Mesodermal Baculovirus (SEMBV) (Wongteerasupaya, Vickers, Sriurairatana, Nash, Akrajarn, Boonsaeng, Panyim, Tassanakajon, Withyachumnarnkul, and Flegel 1995b). The virus was first characterised as WSSV from an outbreak in a *Penaeus japonicus* in Japan in 1993 (Flegel 1997). The naming of the virus derived from the gross examination displaying white spots. However, Chou, Huang, Wang, Chiang, and Lo (1995) reported that the first epizootic of WSSV occurred in Taiwan in 1992. The dual infection of WSSV and YHLV has since been reported in India and Taiwan (Mohan, Shankar, Kulkarni, and Sudha. 1998; Wang and Chang 2000)

Both WSSV and YHLV can cause significant mortalities in penaeid prawns (Chantanachookin *et al.* 1993; Liu, Wang, Tian, Yin, and Kwang 2002) and are currently the most serious diseases threatening the prawn farming industry in Thailand (Flegel *et al.* 1997). At present there is no further information on the interaction between YHLV and WSSV in cultured prawns. Wang and Chang (2000) suggested that the reason for mass loss in the prawn culture industry in Taiwan between 1996 and 1999 was not only WSSV, but a dual infection of WSSV and YHLV. Prawns with dual infection, generally exhibit only typical signs of white spot syndrome with the YHLV symptoms being less obvious. This would result in the mixed disease being diagnosed as only WSSV infection.

In 1994, prawn farms in northern Australia experienced increased mortality rates in 12 to 15 g prawns, with mortality reaching as high as 80% in some ponds (Owens *et al.* 1998). This disease outbreak was dubbed mid-crop mortality syndrome (MCMS). An investigation into the syndrome initially revealed two distinct viral types using TEM (Owens *et al.* 1998). The two viruses that were implicated as being involved in MCMS were YHLV and SMV (Anderson and Owens 2001). SMV is a

parvo-like virus which had been first identified by Fraser and Owens (1996). This virus was isolated from prawns affected with MCMS by Owens *et al.* (1998). Using a bioassay, Owens *et al.* (1998) reported that this parvo-like virus was capable of causing mortality. The gross symptoms of SMV were lethargy, reduced feeding, and redness of the carapace and pleopod (Fraser and Owens 1996). Owens *et al.* (1998) reported that SMV virulence in MCMS affected prawns was enhanced by the presence of other coinfecting viruses such as an enveloped, filiform virus. The coinfecting virus was YHLV, which had first been identified by Spann *et al.* (1995). The enhanced virulence from YHLV was demonstrated when Owens *et al.* (1998) treated prawn extract with ether prior to injecting it into *P. monodon*. As SMV is un-enveloped, the ether should not have harmed the parvovirus. The extract killed at a slower rate than untreated extract, suggesting that the SMV was capable of causing mortality but that its virulence was enhanced by the presence of other coinfecting viruses such as an enveloped, filiform virus like YHLV. Spann *et al.* (1997a) reported that YHLV was also isolated from prawns affected by MCMS. They reported that diseased prawns were observed swimming at the surface and edge of ponds and displayed varying degrees of red body colouration. Symptoms from both of these viruses were apparent in MCMS affected prawns.

In recent years, two other viruses have been reported being present during the MCMS. These viruses are IHHNV (Krabsetsve *et al.*, 2004) which is similar to SMV with respect to being a non-enveloped DNA virus and MoV (Cowley *et al.*, 2005) which is similar to YHLV with respect to being an enveloped RNA virus. These two viruses could both have influenced the mortality of the infected prawns.

In 2000, Smith reported that lesions were found in a *P. monodon* displaying non-specific signs of disease. He reported that the causative agents of lesions appeared to be *Vibrio* spp. and a rod shaped virus similar to YHLV. With the use of TEM it was observed that the nerve cells in the fasciculated zone contained cytoplasmic vesicles with particles and rod shaped nucleocapsids. These rods were similar to YHLV and were 130 to 260 nm long by 10 to 16 nm in diameter and had a helical symmetry with a screw like thread. Also an unidentified enveloped virus, ranging from 50 – 96 nm in diameter, was observed in cytoplasmic vesicles in the fasciculated zone (Figure 2.6). Smith (2000) reported that the unidentified enveloped virus was a

possible aetiological agent in one of the disease outbreaks. In the paper, Smith (2000) did not suggest what the virus was. It is likely that this virus was MoV which is an enveloped virus, averaging 85 – 100 nm in diameter. From the TEM photograph (Figure 2.7), Smith (2000) also reports particles of 20 nm in diameter. However, Smith (2000) does not suggest to what these could be. It is highly likely that these particles were the parvovirus SMV which is reported to be non-enveloped, averaging 20 nm in diameter. This would suggest triple viral interaction between YHLV, SMV and MoV to cause disease.



Figure 2.6. TEM of vesicle showing particles (PAR) of 50 - 96 nm in diameter and rod-shaped structures (ROD) 155 – 207 nm long (Smith 2000).

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Figure 2.7. TEM of vesicles within nerve cells of the fasciculated zone of the eye of moribund *Penaeus monodon*. The vesicles are 3 μm in diameter and contain unidentified particles (PAR) of 20 nm in diameter. Some particles also appear to be free in the cytoplasm (Smith 2000).

2.7 Yellow head-like infections in other crustaceans

A range of crustaceans can be infected with YHLV (Table 2.1). *P. monodon* is the only crustacean that is commonly affected by YHLV (Walker *et al.* 2001). However, several other penaeid prawns and other crustaceans have been reported to be susceptible by either natural or experimental infection. Flegel (1997) reported that, unlike WSSV, YHLV has not been seen to infect crabs or freshwater prawns and that the wide spectrum of hosts within the penaeid species suggests there is a common mechanism of infection that results in preventative methods being hard to develop. Natural infection from YHLV has only been detected in *Penaeus esculentus* that were co-cultivated with *P. monodon* (Table 2.1). However, only 14 prawns from one location were tested with 8 being positive. For a more substantial conclusion to be drawn, larger sample numbers are needed. The YHLV infection in *P. esculentus* was reported to be chronic and there were no signs of gross disease. In Thailand, it has

been reported that co-cultivation of *Penaeus merguensis* and *Penaeus indicus* with *P. monodon* during early outbreaks of YHLV resulted in no disease in the *P. merguensis* and *P. indicus* (Limsuwan 1991, Mohan *et al.* 1998).

Spann *et al.* (2000) reported that different penaeids have different susceptibility to YHLV infection. The authors demonstrated that *P. monodon*, *P. japonicus*, *P. esculentus* and *P. merguensis* were susceptible to experimental infection. The results showed that *P. monodon* were the most susceptible out of the four different penaeids and that *P. japonicus* were the least susceptible and displayed a size related response to the disease with an increased size resulting in increased resistance. Spann *et al.* (2000) suggested that the difference in susceptibility between the penaeids is likely to be related to both the dose and their relative susceptibility to the disease, rather than an indication of resistance to infection. The experiment by Spann *et al.* (2000) has demonstrated that the four mentioned penaeids were susceptible to infection by YHLV via intra-muscular injection. However, this does not demonstrate that they can be naturally infected. For more substantial results, the prawns should have been infected via bath inoculum or fed infected carcasses. This would give a stronger indication as to whether the three species of prawns are naturally susceptible to YHLV infection because the viral agent would not be by-passing the primary defenses of the prawn, i.e., the cuticle or gut.

Table 2.1. Natural and experimental host range for YHLV (Flegel 1997, Walker *et al.* 2001).

Species	Evidence of Infection YHLV
<i>Penaeus monodon</i>	N, E
<i>Penaeus esculentus</i>	N, E
<i>Penaeus duorarum</i>	E
<i>Penaeus japonicus</i>	N, E
<i>Penaeus merguensis</i>	E
<i>Penaeus aztecus</i>	E
<i>Penaeus setiferus</i>	N, E
<i>Penaeus stylirostris</i>	E
<i>Penaeus vannamei</i>	E
<i>Metapenaeus enis</i>	N
<i>Metapenaeus bennettiae</i>	E
<i>Palaemon styliferus</i>	N
<i>Euphasia superba</i>	N

N - Natural infection

E- Experimental infection

2.8 Signs of infection and detection methods of YHLV

Initially, infection of YHLV in *P. monodon* of cultured populations and experimental trials affected juvenile to sub-adult prawns (5 to 15 g) and often induced 100 % mortality within three to five days from the infection date (Chantanachookin *et al.* 1993). However, natural disease outbreaks have been reported in *P. monodon* up to 40 g (Spann *et al.*, 1997). The first gross sign of infection is an increase in feeding at an abnormally high rate for several days followed by a sudden drop in appetite (Chantanachookin *et al.* 1993). Within one day of the prawns ceasing to feed, they begin either slowly or erratically swimming near the edge of the pond, and mortalities soon follow. Dead prawns are found at the edge of the pond and scattered evenly over the entire bottom of the pond. The disease is usually characterized by a pale to yellowish colouration of the cephalothorax and gills due to the underlying

yellow hepatopancreas showing through the translucent carapace of the prawn and also a generally pale or bleached appearance of affected prawns (Chantanachookin *et al.* 1993, Cowley *et al.* 1999). It has also been reported that YHLV from Australia causes the colour of the prawn to change to a degree of pink to red, with primarily the appendages, tail fan and mouth parts being most noticeable. Spann *et al.* (1997) also reported that the gills changed from the normal clear/yellow, to pink and the prawns exhibited fouling of the gills, and shell, and tail rot. However, with my experience working on prawn farms checking prawns for disease, I have not seen evidence that gill fouling is worse in prawns expressing the YHLV disease compared to prawns not exhibiting the disease.

Spann *et al.* (1997) reported that with the use of a light microscope, diseased prawns display disorganisation and loss of normal, defined tubule structure in the lymphoid organ. The gills of diseased prawns displayed structural damage such as fusion of gill filament tips, general necrosis and loss of cuticle from primary and secondary lamellae. However, this detection method and signs of disease only indicate some form of disease and as mentioned previously, these symptoms are also characteristic of penaeid prawns infected with other different viruses.

Flegel *et al.* (1997) reported that YHLV can be diagnosed histologically in moribund prawns by the presence of intensely basophilic inclusions in many different tissues and that these inclusions can be seen best with haematoxylin and eosin (H&E) staining of sectioned stomach and gill tissue. However, Chantanachookin *et al.* (1993) reported that there is no clear way to characterise YHLV infection using standard histological examination with H&E-stained preparations. This is because the cytoplasmic, virus-associated inclusions stain deeply basophilic in the same manner as pyknotic nuclei and it is difficult to differentiate between the two without the use of an electron microscope. Chantanachookin *et al.* (1993) also reported that the lymphoid organ was distinctly abnormal in all YHLV infections, with nuclear abnormalities, cytoplasmic abnormalities and necrotic cells. These symptoms are also characteristic of penaeid prawns infected with other viruses.

The TEM which gives an image of the virus is the gold standard test for the detection of YHLV infection e.g. Spann *et al.* (1997) used TEM to detect YHLV infection.

However, this method is not useful for detecting early stage infections or for on-farm application. It is time consuming, requires expensive equipment and requires the prawns to be killed, and therefore, it is not applicable for on-farm use or for screening hatchery broodstock.

In 1997, Wongteerasupaya *et al.* developed a RT-PCR for the detection of YHLV in Thailand. This test was specific and sensitive for the selected region of the YHLV genome, with other nucleic acid templates (WSSV and HPV) giving no amplification signal. The RT-PCR was able to amplify as little as 0.01 pg of YHLV-RNA and showed evidence of infection in *P. monodon* at 6 to 12 hours (h) after experimental exposure to the virus. However, it is more likely that the RT-PCR detected YHLV genome that was injected into the prawn and it was not detecting infection at such an early time after injection. In 2000, Cowley *et al.* (2000b) developed an RT- nPCR for the detection of YHLV within Australia. This test is a rapid, specific and highly sensitive method for detecting YHLV in prawn tissue. Using the RT-PCR technique, the amplification is able to detect 0.01 pg cDNA while using the RT- nPCR technique 0.01 fg cDNA was detectable. The YHLV genome was able to be detected within 6 h of experimental infection of a *P. japonicus*. However, again it is probable that the test was detecting genome that was injected into the prawns and does not necessarily indicate replication. Gill biopsies can be used as a tissue sample (Cowley *et al.*, 2000) or more recently, it has been reported that dried haemolymph can be used for the detection of YHLV with RT-PCR (Kiatpathomchai, Jitrapakdee, Panyim, and Boonsaeng 2004), resulting in the ability to sample broodstock. As mentioned before, this technique has practical limitations for widespread commercial application, requiring specialised equipment and highly trained personnel, resulting in expensive assay costs. A multiplex RT- nPCR has been developed for the differentiation of YHLV from Australia and YHLV from Thailand (Cowley, Cadogan, Wongteerasupaya, Hodgson, Boonsaeng, and Walker 2004). The test detected the YHLV in approximately 10 fg of lymphoid organ total RNA. The YHLV from Australia produced a 406 bp product while the YHLV from Thailand produced a 277 bp product.

Real time PCR has been developed for the detection of YHLV (Dhar, Roux, and Klimpel 2002; Vega *et al.*, 2004). Both of these tests have the same sensitivity as the RT-PCR's but give quantitative load of infection in the sample.

In situ hybridisation has been developed (Tang and Lightner 1999) for the detection of YHLV infection in *Penaeus vannamei* using a cDNA fragment labelled with digoxigenin that resulted in a highly sensitive test. This same probe was subsequently used to detect YHLV from Australia (Tang, Spann, Owens, and Lightner 2002). Spann, McCulloch, Cowley, East, and Walker (2003) have also reported the development of an *in situ* hybridisation probe for the detection of YHLV in *P. monodon* and *P. esculentus* within Australia. However, these molecular techniques have practical limitations for widespread commercial application. This includes the need for special equipment and highly trained personnel, which result in expensive assay costs for small sample numbers that can limit RT-PCR and *in situ* hybridisation use.

Polyclonal antisera have been produced for the detection of YHLV (Nadala *et al.* 1997). However, the assay (Western blot analysis) was not highly sensitive and was not applicable to on-farm field examinations (Sithigorngul, Rukpratanporn, Longyant, Chaivisuthangkura, Sithigorngul, and Menasveta 2002). Reported in 2000 and again in 2002, Sithigorngul *et al.* produced monoclonal antibodies (MAbs) specific to YHLV enveloped protein. In both experiments, IgG-MAbs were produced. The first experiment resulted in a low yield of hybridomas with MAbs specific to YHLV due to the usage of crude YHLV extract from gills of an infected *P. monodon*. The second experiment resulted in higher success of hybridomas specific to YHLV. Sithigorngul *et al.* (2002) reported that most of the YHLV specific MAbs were specific to the 67 kDa protein and only a few were specific to the 135 and 22 kDa protein. Sithigorngul *et al.* (2002) also reported that several antibodies against YHLV did bind to haemolymph and tissues from uninfected prawns. He did not elaborate as to why this may have occurred. There could be three possibilities for this occurrence; non-specific binding to similar epitopes from the prawn cells, the hybridomas not being sufficiently screened, or the most probable reason, that the reported uninfected prawns had an undetected low level of infection.

A paper by Nadala *et al.* (1997) reported that YHLV haemagglutinates chicken erythrocytes. They used this HA activity as a qualitative and quantitative detection method for YHLV. Munro and Owens (2005) used this HA activity of the YHLV to develop a low cost detection method for YHLV in *P. monodon*, while they demonstrated that YHLV RT-nPCR negative prawns caused negligible HA.

2.9 Conclusion

The world prawn aquaculture industry is a rapidly increasing industry, being one of the fastest growing aquaculture sectors in Asia and Latin America. In 2004 the prawn aquaculture industry produced 2.47 million tons valuing, US\$ 9735 million (Figure 2.8), compared to 1.65 million tonnes produced in 2000, equating to a 50% increase in production within 4 years.

The Australian prawn farming industry now produces over 4,000 tonnes of product annually with a farm gate (producer) value in excess of AU\$70 million, providing more than 1000 direct jobs and 1800 indirect jobs. The Australian industry is one of the smaller volumetric producers in the world but leads the world in productivity with an average yield of more than 4,500 kg per hectare. Prawn farming is now one of the largest aquaculture sectors in Australia behind pearl oysters, tuna and salmon.

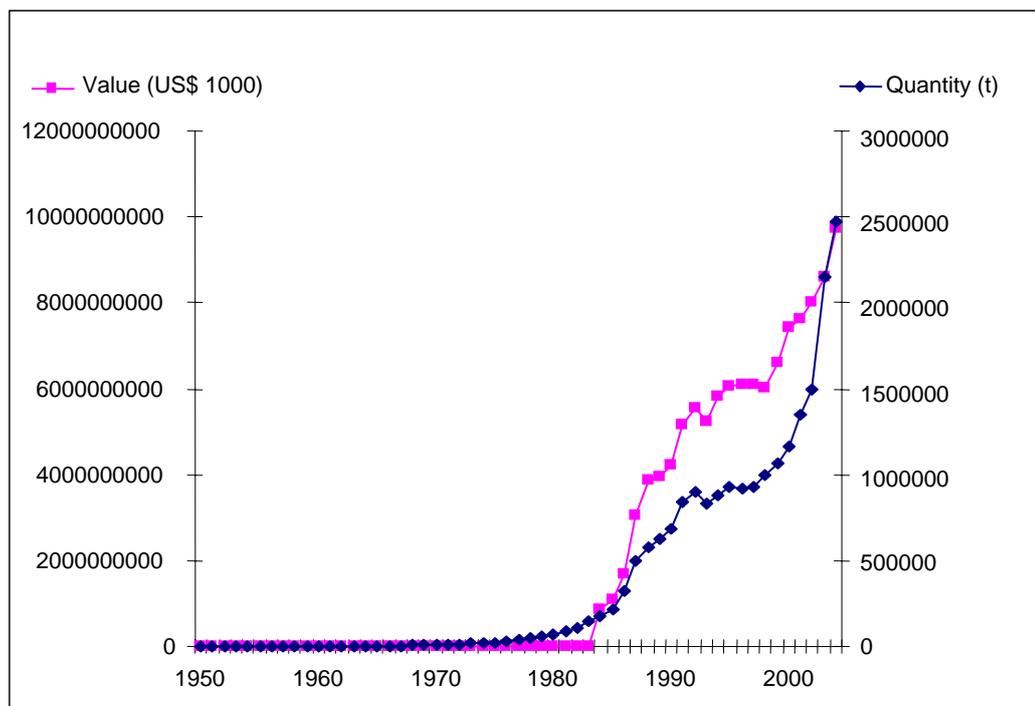


Figure 2.8. The world prawn aquaculture production and value up to 2004 (FAO).

Infectious diseases, particularly viral diseases are recognised as the major threat to the long-term viability of the prawn farming industry worldwide. The major taxonomic groups are the families *Nimaviridae*, *Parvoviridae*, *Picornaviridae* and the order *Nidovirales*. Over the last ten years, widespread epidemics from viruses have affected all aspects of prawn farming, from intensive farms in Thailand to extensive systems in Bangladesh.

The emergence of 'new' viruses is rapidly increasing. For example, in 1990 Lightner *et al.* reported six viruses affecting penaeid prawns. By 1992, the list of known viruses affecting penaeid prawns had increased to 12 (Lightner 1996) and presently, around 18 viruses have been reported in penaeid prawns.

At present, the most common methods for the detection of prawn viruses use PCR or specific probes. These diagnostic methods are sensitive and specific for the detection of viruses, however, they require special equipment / reagents and highly trained personnel, which result in expensive assays and thus a limited number of samples being tested, which prevents a lot of farms in developing countries from testing any of their stock. This results in increased spread of disease and increased mortalities on the farm due to unknown viral infection or viral load of the prawns being produced or grown in hatcheries and farms. For this reason cheaper detection methods such as the antibody based tests or HA need to be available for on-farm application so the farmer can detect the presence of the virus and the loading of that virus in their farm/broodstock.

As previously mentioned, when a viral epidemic occurs, one virus is usually assigned as the aetiological agent. This is because either only the most probable virus was tested for or if other viruses were detected but were in low numbers then they are usually ignored and the virus in the greatest abundance is designated as the disease causing agent. With the high level of prawn viral infection throughout the world, a single viral infection is not very probable. At present there are no reports on the effects of dual infection of viruses in prawns with regards to disease. For a greater understanding of how prawn viruses are interacting with each other to cause disease, there needs to be further research to infect prawns with individual viruses and

compare them with prawns dually infected with known viruses. This would enable farmers to predict the likelihood of a disease outbreak occurring. For example, it has been previously reported that YHLV infection is present in approximately 97 % of *P. monodon* in Australia. It would be an advantage to determine the effect of dual infection of SMV, MBV, IHHNV, MoV etc in the same prawns to determine the likelihood of disease occurring and what mixture of viruses result in disease expression.

The process by which viruses cause disease is not yet understood as not all prawns with a specific virus will express the disease and suffer mortality. Disease appears to occur when the prawns are not able to control the covert form of infection. There is no report that indicates a genetic change (i.e. mutation) of the virus is required to cause disease. There are many possible triggers for diseases to appear, some of these are external factors such as environmental stress (i.e. poor water quality), in some viruses the size and age of the prawn seems to determine the likelihood of disease outbreak (i.e. IHHNV), or a secondary, complicating infection.

In healthy prawns, the virus is primarily contained within partitioned areas of lymphoid organ tissue (spheroids). The virus is also commonly present in other tissues including spermatophores. Generally, the virus is at very low levels and appears to multiply very slowly. In diseased prawns, there is an explosion of multiplication in the lymphoid organ and the virus rapidly invades other tissues in the prawn. The prawn is unable to contain the infection, resulting in it becoming sick and mortality usually follows.

The following research in this thesis investigates YHLV as the disease causing agent on Australian prawn farms and explores alternative detection methods for the detection of YHLV that are applicable to prawn farms within Australia and developing countries. Due to YHLV being imbedded in the current literature as either GAV or YHV, the following research will refer to these viruses as individual viruses.

CHAPTER 3

DEVELOPMENT OF A SYNTHETIC POSITIVE CONTROL WHICH DETECTS PLASMID CONTAMINATION IN DIAGNOSTIC POLYMERASE CHAIN REACTION FOR GILL-ASSOCIATED VIRUS

3.1 Introduction

The use of polymerase chain reaction (PCR) has become a routine method for the detection of a vast range of genomic material. This technique is highly sensitive and specific for the amplification of target genome. While using this assay, it is routine to use a known positive sample to ensure the assay performed correctly, minimising false negatives. Generally, the PCR positive control is derived from original positive samples cloned into a plasmid, giving reproducible results. Due to the high concentration of plasmid positive controls and the duration that they are used in the laboratory, contamination of diagnostic samples is always a concern and once contamination has occurred it is impossible to identify because it will have the same sequence as the clinical samples. When large numbers of samples are being screened, it is crucial that contamination can be easily identified as unidentified false positives will severely affect the accuracy of the results.

This chapter describes a method of producing a plasmid positive control for use in a PCR for the detection of gill-associated virus (GAV) that has a different sequence than GAV and gives a noticeably different size on agarose gel, while still using the same specific primers and PCR conditions as used for the amplification of the GAV genome. The intended use of this plasmid positive control is to confirm that the PCR worked correctly when screening large numbers of prawns from different farms to examine the effect of GAV on production.

3.2 Material and Methods

3.2.1 Diagnostic primers

The diagnostic primers described in this chapter are GAV-5 (AAC TTT GCC ATC CTC GTC AC-3'), GAV-6 (5'- TGG ATG TTG TGT GTT CTC AAC-3') for the outer nest, and GAV-1 (5'- ATC CAT ACT ACT CTA AAC TTC-3') and GAV-2 (5'- GAA TTT CTC GAA CAA CAG ACG-3') for the inner nest (Cowley *et al.* 2000b). The primers GAV-5 and GAV-6 amplify a 618 base pair (bp) product and the primers GAV-1 and GAV-2 amplify a 317 bp product internal to the region amplified by primer GAV-5/GAV-6 from a total sequence of 26235 nucleotides from the GAV genome (Cowley and Walker 2002). The reverse transcription nested polymerase chain reaction (RT-nPCR) was performed as previously described by Cowley *et al.* (2000b).

3.2.2 PCR plasmid positive control

P1 (5'- ATC CTC GGC ACC GTC A – 3') and P2 (5' - AAG CAG CCC AGT AGT AGG TTG – 3') were designed to amplify a 509 bp region of a 3010 bp pBR322 plasmid. The oligonucleotide P1 was then theoretically attached to primer sequence GAV-2 and the oligonucleotide P2 was then theoretically attached to primer sequence GAV-1. This gave a oligonucleotide sequence of GAV-2/P1 (5'- GAA TTT CTC GAA CAA CAG ACG ATC CTC GGC ACC GTC A – 3') and GAV-1/P2 (5' - ATC CAT ACT ACT CTA AAC TTC AAG CAG CCC AGT AGT AGG TTG – 3). This product gave a theoretical product size of 551 bp when using the inner nested primers for GAV (234 bp larger than the diagnostic nested sample). To design the outer nested product with the outer primers for the target sequence which gave a larger amplified product than the clinical samples, 40 bp of strawberry pectin esterase sequence randomly chosen from GenBank (Acc: CB934832) was theoretically attached to each of the 5' ends of the primers GAV-2/P1 and GAV-1/P2. The two strawberry pectin esterase sequences were S1 (5'-GGATGTTGTGTGTTCTCAACCAACTCCGCGGTAATAAAA-3') and S2 (5'-TCTGATGCTAGCTCTGCCTATTCTCCGGTTGCTTCTTAAC – 3') respectively. The outer primers (GAV-6 and GAV-5) were then theoretically

attached to the 5' end of the oligonucleotide. This gave a sequence of, GAV6/S1/GAV2/P1 (5'-TGGATGTTGTGTGTTCTCAACCAACTTCCGCGGTAATAAACAGGTGTTCCAACGTTTCATGGGAATTTCTCGAACAAACAGACGAA GCAGCCCAGTAGTAGGTTG-3') and GAV5/S2/GAV1/P2 (5'-AACTTTGCCATCCTCGTCACTCTGATGCTAGCTCTGCCTATTCTCCGGTTGCTTCTTAACATCCATACTACTCTAAACTTCATCCTCGGCACCGTCA-3') respectively. This gave a theoretical product size of 672 bp (54 bp larger than diagnostic PCR product) (Figure 3.1). The sequence of the two theoretically designed primers; GAV6/S1/GAV2/P1 and GAV5/S2/GAV1/P2 were synthesised at 0.2 μ mol synthesis scale by Sigma Genosys Australia Pty. Ltd.

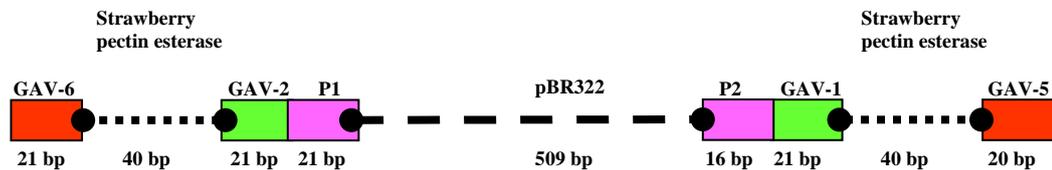


Figure 3.1. A diagrammatic representation of the oligonucleotide developed for the PCR positive control using primers developed for the detection of gill-associated virus.

The optimised PCR conditions (final concentration in 50 μ l total volume) for amplification of the positive control oligonucleotide were: primers (2.5 μ M each), dNTPs (200 μ M) (Progen), Proof start DNA polymerase (2.5 U) (Qiagen), in 10 \times PCR buffer. Three microliters of template (pBR322) (100 μ g/ μ l) was used in the reaction. The optimised PCR conditions were eight cycles of 95 $^{\circ}$ C/5min, 94 $^{\circ}$ C/1 min, 60 $^{\circ}$ C/ 30 s and 68 $^{\circ}$ C/ 1 min, followed by 26 cycles of 94 $^{\circ}$ C/50 s, 68 $^{\circ}$ C/1 min, followed by 68 $^{\circ}$ C/ 5 min and a 15 $^{\circ}$ C hold using an Eppendorf gradient thermocycler. This ramp-up style of PCR was used to initially focus on the two single primers specific for the pBR322 template which required a lower annealing temperature than the entire oligonucleotide (16 – 21 bp compared to 97-103 bp). Then secondly, once the primers specific to pBR322 had started to bind to the template, the annealing temperature was increase to 68 $^{\circ}$ C for the complete primer sequence. This higher temperature reduced non-specific binding that would normally be expected of such large primers. Once the PCR was completed, the product (20 μ l) was resolved in 2%

agarose-TAE gels containing $0.5 \mu\text{g ml}^{-1}$ ethidium bromide. This product was then visualised under ultraviolet light and the product was excised from the agarose gel and contaminants were removed with the use of Wizard SV Gel and PCR clean-up system (Promega). This purified product was then cloned into *Escherichia coli* JM109 cells. The *E. coli* JM109 cells which were successfully transformed (via blue/white screening) were screened for the correct insert using the diagnostic primers for GAV. The *E. coli* JM109 colonies that were positive for the correct insert were stored at -80°C in 10% glycerol.

3.3 Results

3.3.1 Plasmid positive control

It can be seen that the PCR product of the positive control was 54 bp larger in the outer nest and 234 bp larger in the inner nest compared to the GAV samples (Figure 3.2).

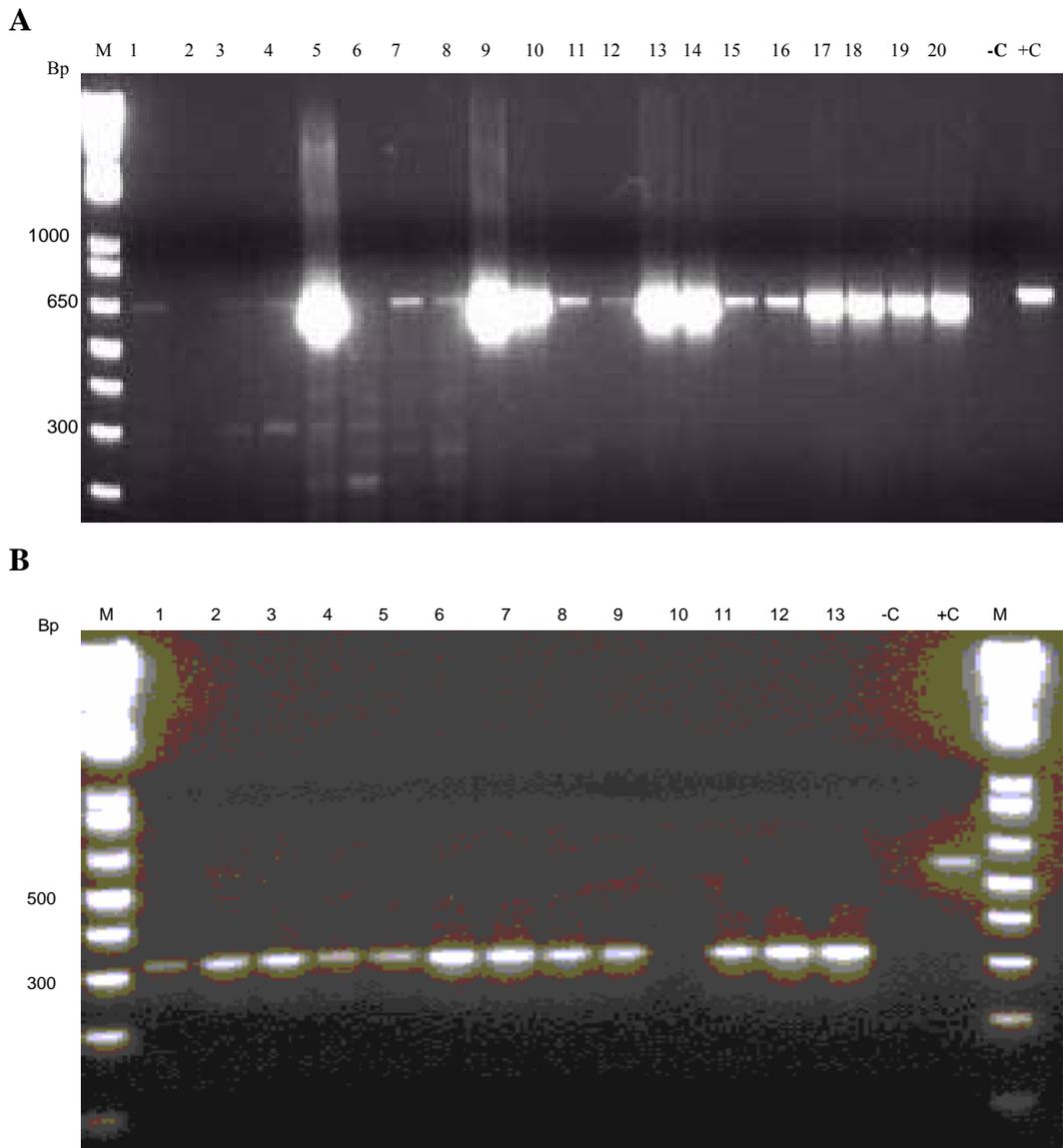


Figure 3.2. RT-PCR (A) and RT-nPCR (B) of clinical samples of *Penaeus monodon*.
A: RT – PCR products (618 bp), using primers GAV5 and GAV6 (lanes 1 – 20)
B: RT – nPCR products (317 bp) (lanes 1 – 13), using primers GAV1 and GAV2.
(M) molecular weight marker, (-C) negative control, (+C) plasmid positive control.
Note: Figure 3.2 A & B are not from the same prawn samples.

3.4 Discussion

This chapter described a simple technique to develop a positive control for diagnostic nested PCR. Developing the PCR product to be noticeably larger than the diagnostic product in both the outer and inner nest enables easy identification of contamination by plasmid control, which in turn reduced false positives. This PCR positive control (when optimised) can be used as an internal PCR control as it is larger than the diagnostic samples it should not overly compete in the PCR reaction. The use of an internal PCR control has been demonstrated by Helweg-Larsen Jensen, Benfield, Svendsen, Lundgren, and Lundgren (1998), who reported that the diluted (10 fold) internal control produced no increase in the detection limit of their plasmid positive control for the diagnostic sample. It is advisable that the insert used to increase the size of the outer nest be of material not used in the laboratory, in the case for the aquatic pathology laboratory at James Cook University, human genome or crustacean genome was avoided.

The ramp-up PCR conditions used in this chapter prior to cloning the product, enabled a 103 bp oligonucleotide to be used, while not causing non-specific binding. The method of working up to a high annealing temperature could be used for any PCR application using large oligonucleotide primers.

The main importance of this nested PCR positive control is that once the positive control is added at the set-up of the outer nested PCR, no other additional positive control material needs to be added for the nested PCR. This gives a positive control to show that the PCR has indeed worked correctly throughout the two PCR reactions. This results in reduced contamination and a one step addition of the PCR control for a two step PCR reaction, all of which reduces false positives that can cause questionable results of the clinical samples.

CHAPTER 4

IS GILL-ASSOCIATED VIRUS RELATED TO DECREASED PRODUCTION OF *Penaeus monodon* IN AUSTRALIAN PRAWN FARMS

4.1 Introduction

Gill-associated virus (GAV) is the type species of the *genus Okavirus* in the *family Roniviridae* of the *Nidovirales* (Mayo, 2002) and is the junior synonym of lymphoid organ virus (LOV) which was reported in 1995 by Spann *et al.* LOV was reported to be found only in the lymphoid organ and was similar to yellow head virus (YHV) with respect to ultrastructural and cytopathological features. However, LOV had no association with disease or mortality (Spann *et al.*, 1995; Spann *et al.*, 1997). GAV was subsequently reported as a pathogenic relative of LOV, found both in the lymphoid organ and the gills of infected *Penaeus monodon* (Spann *et al.* 1997). Using sequence analysis it was determined that GAV and YHV share 85.1% sequence identity indicating that they are geographic topotypes (Cowley *et al.*, 1999). With the development of a highly sensitive reverse transcription nested polymerase chain reaction (RT-nPCR) it was reported that LOV has a 98.9% similarity to the GAV sequence, indicating that they are the same virus (Cowley *et al.*, 2000).

From a sample size of 148 prawns, the reported prevalence of GAV infection in *P. monodon* broodstock captured in northeastern Queensland is 97.3%. From a sample size of 50, the prevalence of GAV in postlarvae was 100% and from a sample size of 56 the prevalence of GAV in juveniles was 98.2% (Walker *et al.* 2001). However, it is not reported how these samples were obtained, so it is unknown if they are from the same hatchery and/or the same broodstock.

Despite the high prevalence of GAV, not all GAV-infected *P. monodon* express disease. For GAV-related disease to be expressed, there appears to be other factors involved. These are hypothesised to be the viral load of parental broodstock, the initial viral load of postlarvae or an unknown environmental factor acting as a stressing agent.

GAV has been reported to be highly virulent to *P. monodon*. The pathogenicity of GAV was determined by inoculation of filtered homogenates of lymphoid organ, gills and whole cephalothoraces from *P. monodon* that were positive for GAV, resulting in mortality from 7 to 8 days (d) post-inoculation (Spann *et al.*, 1997). However, at the time of the pathogenicity trial there were at least five other viruses infecting Australian farmed *P. monodon*. These viruses consisted of monodon baculovirus (Doubrovsky *et al.*, 1988), lymphoid parvo-like virus (Owens *et al.*, 1991), infectious hypodermal and hematopoietic necrosis virus (Owens *et al.*, 1992, Krabsetsve *et al.*, 2004), spawner-isolated mortality virus (Fraser and Owens 1996) and Mourilyan virus (Cowley *et al.*, 2005) which all could have possibly influenced the mortality of the *P. monodon* in that trial. This infection trial was repeated by Vega *et al.* (2004), where the prawns were again inoculated with filtered prawn homogenate containing levels of GAV (not purified virus). They reported that 100% (15/15) of the GAV-injected prawns died compared to 40% (2/5) of the controls while there was a significant increase ($P = 0.010$) in GAV for both the infected prawns and the controls. However, the GAV increase was significantly higher ($P = 0.047$) in the GAV-injected prawns than the control prawns. Again, no other viruses were tested for in that study or referred to as potential pathogens.

Currently, there are approximately twenty research papers describing different aspects of GAV and only one of those papers attempts to determine if GAV is associated with causing an economic impact on Australian prawn farms. Callinan *et al.* (2003a) reported that a yellow head-related virus such as GAV was causally associated with mortality on an Australian prawn farm.

This current study investigates the association of GAV with survival on three prawn farms located over three different geographic locations along the east coast of Australia, to determine whether GAV in *P. monodon* is associated with disease and mortality within Australian aquaculture.

4.2 Materials and Methods

4.2.1 Purification of gill-associated virus

Methods used to purify GAV were adapted from Wongteerasupaya *et al.* (1997) and Nadala *et al.* (1997). Fifty grams of gill tissue of *P. monodon* were homogenised in 500 ml (1 in 10 w/v) of TNE buffer (0.02 M Tris HCl, 0.2 M NaCl, 0.02 M EDTA pH 7.4) and centrifuged at $5000 \times g$ for 10 min (Eppendorf centrifuge, A-4-44 rotor). The precipitate was discarded and the supernatant was centrifuged again at $5000 \times g$ for 30 min (Eppendorf centrifuge A-4-44 rotor). The supernatant was filter sterilised through a 0.45 μm filter and centrifuged at $100\,000 \times g$ for 1 h (Beckman ultracentrifuge, Ti 70.1 rotor). The resulting pellet was resuspended in TNE buffer and overlaid onto a 25 – 55 % sucrose gradient and centrifuged at $100\,000 \times g$ for 20 h (Beckman ultracentrifuge, SW40 rotor). After centrifugation, viral bands were visualised by top down illumination, and removed using an 18-gauge needle and diluted 5 times in TNE before centrifugation at $100\,000 \times g$ for 1 h. The resulting pellet was resuspended in 500 μl of phosphate buffered saline (0.1 M phosphate, 0.138 M NaCl, 2.07 mM KCl, pH 7.2) (PBS) and stored at -20°C .

4.2.2 RNA isolation

RNA isolation was performed using TRIzol (Life Technologies Pty. Ltd.) as described by the manufacturer's instructions using the options for small RNA quantities.

4.2.3 Serial dilution of GAV RNA

GAV RNA was serially diluted 1 in 10 into *Cherax quadricarinatus* (red claw crayfish) total RNA (i.e. 2 μl of GAV RNA into 20 μl of *C. quadricarinatus* total RNA). This sample was diluted thirteen times. The crayfish used in this experiment tested negative for GAV by RT-nPCR.

4.2.4 Reverse transcription - nested PCR

The method to reverse transcribe GAV RNA to cDNA and the following amplification of the cDNA was adapted from Cowley *et al.* (2000).

4.2.5 PCR primers

The primers used in this study for the detection of GAV were developed by Cowley *et al.* (2000). Briefly, the outer nest primers, GAV-5 (5'-AAT TTT GCC ATC CTC GTC AC-3') and GAV-6 (5'-TGG ATG TTG TGT GTT CTC AAC-3') amplified a 618 base pair (bp) region of the genome. The inner nested 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') amplified a 317 bp region of the genome.

4.2.6 Prawn samples from this farm study

Gill tissue from *P. monodon* was obtained by sampling a total of forty five prawn ponds from three prawn farms at both one week poststocking and at one week preharvest over the period of 2002 to 2004. The three prawn farms were situated at different geographic positions along the northeastern coast of Australia to reduce the possibility of location bias. The three locations were: 1) Palmers Island (New South Wales) - 29° 44' South, 153° 26' East; 2) Woongoolba (Queensland) - 27° 73' South, 153° 32' East and 3) Cardwell (Queensland) - 18° 27' South, 146° 02' East.

Individual gill samples was removed from 20 prawns per pond and were collected at one month poststocking then again at one week preharvest from the tested ponds. Gill tissue was snap frozen in liquid nitrogen and then stored at – 80°C prior to processing.

4.2.7 Positive control

To eliminate false positives due to plasmid contamination, a previously developed synthetic positive control (Chapter 3, Munro *et al.*, 2005) was used in all RT-nPCR reactions.

4.2.8 Negative control

Gill tissue from *C. quadricarinatus* was used as a negative control (GAV has never been detected in *C. quadricarinatus* and all *C. quadricarinatus* in this experiment previously tested negative for GAV).

4.2.9 Case definitions

The definition of a GAV-related outbreak was adapted from Callinan and Jiang (2003b), where the following criteria was used:

Affected pond was a pond in which numbers of moribund or dead prawns at the edge increased by at least 10 daily for at least 5 consecutive days. i.e. 10, 20, 30, 40, 50 prawns/day. Thus using this criteria, combined with production data, ponds from the three farms were classified as follows: 1) GAV related outbreak-low production, 2) GAV related outbreak-emergency harvest, 3) No GAV related outbreak-low production, and 4) No GAV related outbreak-high production.

The definition of low production and high production in the selected prawn ponds was as follows:

Low production was where percentage survival was equal to or below 40% at time of harvest.

High production was where percentage survival was greater than 40% at time of harvest.

4.2.10 Grading system

The relative level of infection was derived from the intensities of the visualised outer and inner nested PCR products (10 µl) on a 2% DNA-grade agarose-TAE gel (Progen Industries Ltd) containing 0.5 µl/ml ethidium bromide and visualised under ultraviolet light. It is based on the results that the more intense the visualised band, the more DNA is present which is semi-proportional to the relative amount of viral RNA present in the total RNA. Thus, a larger viral load in the gills means a higher percentage of viral RNA relative to the total RNA and thus an increased amplification of this viral RNA, which leads to a brighter band on the agarose gel. The grading was as follows:

- 4 = strong outer nest (high viral load),
- 3 = weak outer nest (high/medium viral load),
- 2 = strong inner nest (medium/low viral load),
- 1 = weak inner nest (low viral load),
- 0 = no GAV detected (below detection limits).

4.2.11 Statistical analysis

Statistical analysis was performed using Statistical Package of Social Sciences (SPSS) version 12. All data was tested for normality and homogeneity using Q-Q plot analysis and all data was compared using multivariate or univariate analysis of variance (ANOVA) with least significant difference (LSD) post-hoc test and all p-values less than or equal to 0.05 were considered statistically significant.

4.3 Results

4.3.1 Determining if PCR grading system gives a semi-quantitative result

It can be seen that as the initial GAV RNA concentration decreased, so does the intensity of the PCR product using the cDNA (Figure 4.1 A & B). The outer nest PCR product is visually detectable down to the 3rd dilution (Figure 4.1A, lane D). The intensity of the inner nest PCR product can be visually detected out to the 9th dilution (Figure 4.1B, lane J).

The samples in lane A and B were graded 4, while the samples in lane C and D were graded 3, samples in lane E to I (in Figure 4.1B) were graded 2 and the sample in lane J was graded 1 with all other samples being graded 0.

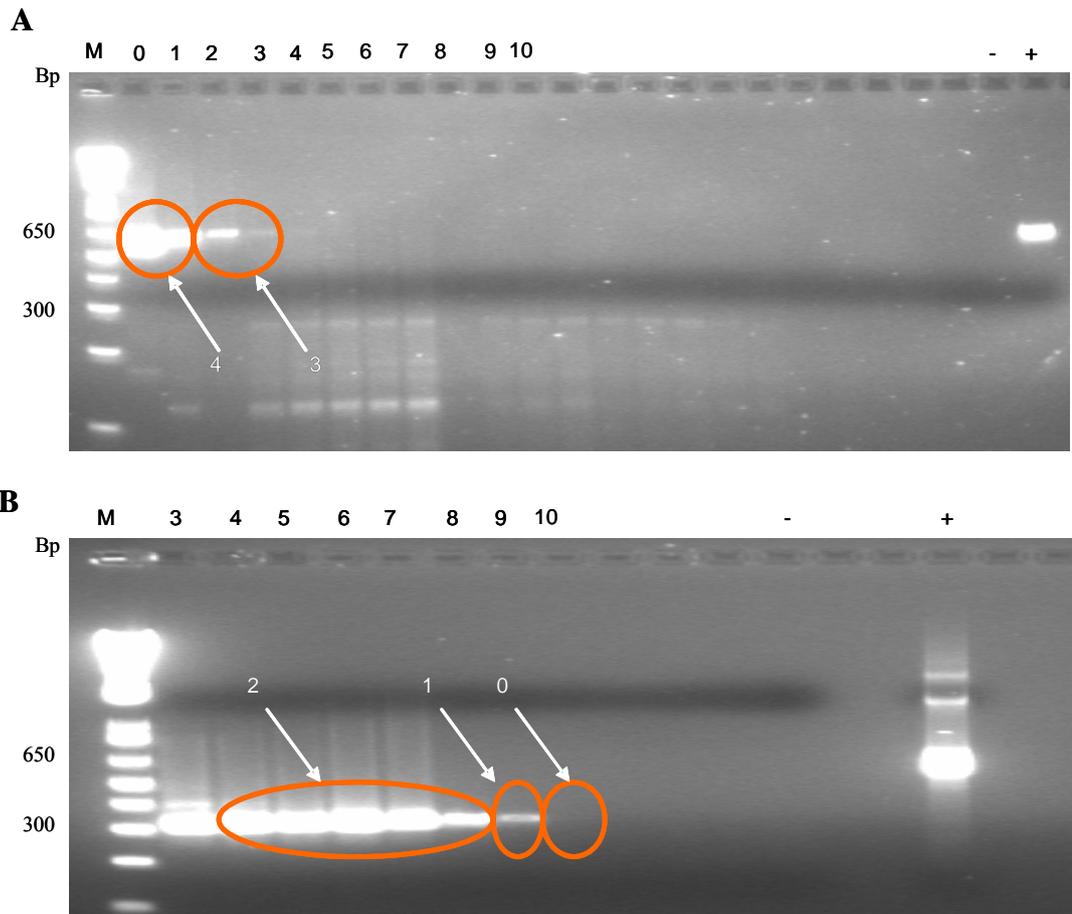


Figure 4.1. RT-PCR (A) and RT-nPCR (B) of RNA of GAV from gills of *Penaeus monodon* 10-fold serially diluted in RNA of *Cherax quadricarinatus*.

A: RT – PCR products (618 bp), using primers GAV5 and GAV6 (lanes A - E)

B: RT – nPCR products (317 bp) (lanes D - J), using primers GAV1 and GAV2.

Note: (M) molecular weight marker, (-) negative control, (+) plasmid positive control

4.3.2 Component two – Determining if GAV has an effect on production

There was an overall significant increase in prevalence and intensity between post-stocking and pre-harvest (sample time) within ponds (Tables 4.1 and 4.2). This indicates that over the production period the intensity and prevalence of GAV increased in all groups of ponds. However, not all groups increased equally between the sampling periods. Ponds with GAV related outbreaks – low production, had an increase in prevalence of 16.2% and an intensity of 41.38 %, the ponds with GAV related outbreak – emergency harvest, had an increase in prevalence of 20.15% and an intensity increase of 42.41%. The ponds which had no GAV outbreak – low production, had a higher increase in prevalence (36.15 %) but a slightly lower increase in intensity (39.04%) than the ponds with a GAV related outbreak. The

ponds with no GAV outbreak – high production, had the lowest increase in prevalence (12.79%) and the lowest increase in intensity (13.77 %) (Table 4.2). This indicates that there was an association of GAV with production i.e. increased prevalence and intensity of GAV results in reduced production. There was also a significant difference between the three farms and the outcome of the crops with respect to prevalence and intensity ($P = 0.004$, $DF = 6, 83$, $F = 3.607$; $P = <0.001$, $DF = 6, 1639$, $F = 7.619$, respectively) indicating that there was a small but significant difference between farms (Table 4.1). This would be expected as the three farms are at different locations. In both cases the F value is relatively small indicating that the location has only a small influence. It can be seen that both prevalence and intensity had an effect on the outcome of the crop (crop status) indicating that both factors had an association with production (Table 4.1). It can also be seen that the two interactions of farm location and sample time, crop status (Farm*Sample time, Farm*Crop status) and the sample time (Crop status*Sample time) all had an effect on the prevalence and the intensity over the production period (Table 4.1). However, these effects were relatively small as can be seen by the F-values.

Table 4.1. The effect of husbandry factors on the prevalence and intensity of GAV over the entire production period. Note: Crop status = GAV-related outbreak-low production, GAV-related outbreak-emergency harvest, No GAV-related outbreak-low production, No GAV-related outbreak-high production. Sample time = poststocking and preharvest.

Source	Prevalence			Intensity		
	DF	F value	Significance	DF	F value	Significance
Farm	2	3.59	0.034	2	6.062	0.002
Crop status	3	20.323	<0.001	3	38.695	<0.001
Sample time	1	99.105	<0.001	1	256.243	<0.001
Farm*Crop status	6	3.607	0.004	6	7.619	<0.001
Farm*Sample time	2	2.148	0.126	2	18.037	<0.001
Crop status* Sample time	3	5.614	0.002	3	29.563	<0.001
Corrected total	83			1639		

Table 4.2. Average prevalence and intensity of GAV in the three farms at one month poststocking, one week pre-harvest and the percentage increase between the sampling periods and percentage daily increase ($n = 1800$).

		GAV-related outbreak-low production	GAV-related outbreak-emergency harvet	No GAV-related outbreak-low production	No GAV-related outbreak-high production
Prevalence (%)	Post-stocking	80.70	75.50	59.38	64.44
	Preharvest	96.30	94.55	93.00	73.89
	% increase	16.20	20.15	36.15	12.79
	% increase/day	0.11	0.14	0.21	0.07
Intensity	Post stocking	1.70	1.48	1.39	1.44
	Preharvest	2.90	2.57	2.28	1.67
	% increase	41.38	42.41	39.04	13.77
	% increase/day	0.28	0.30	0.23	0.07
Survival	%	27.00	52.00	34.00	57.00
Production period	Days	146.00	141.00	173.00	197.00
Pond numbers	Total	15.00	11.00	10.00	9.00

Disease was also associated with both the daily increase in prevalence and intensity and the overall increase in prevalence and intensity over the production period. Ponds that had GAV related outbreak, but low production, had a 0.11% daily increase in prevalence and a 0.28% daily increase in intensity while the ponds that had GAV related outbreaks – emergency harvest had a 0.14% daily increase in prevalence and a 0.30% daily increase in intensity. The ponds with no GAV related outbreak – low production had a 0.21% daily increase in prevalence and a 0.23% daily increase in intensity and the ponds that had no GAV related outbreak – high production had a 0.07 % daily increase in prevalence and a 0.07% daily increase in intensity (Table 4.2 & Figure 4.2).

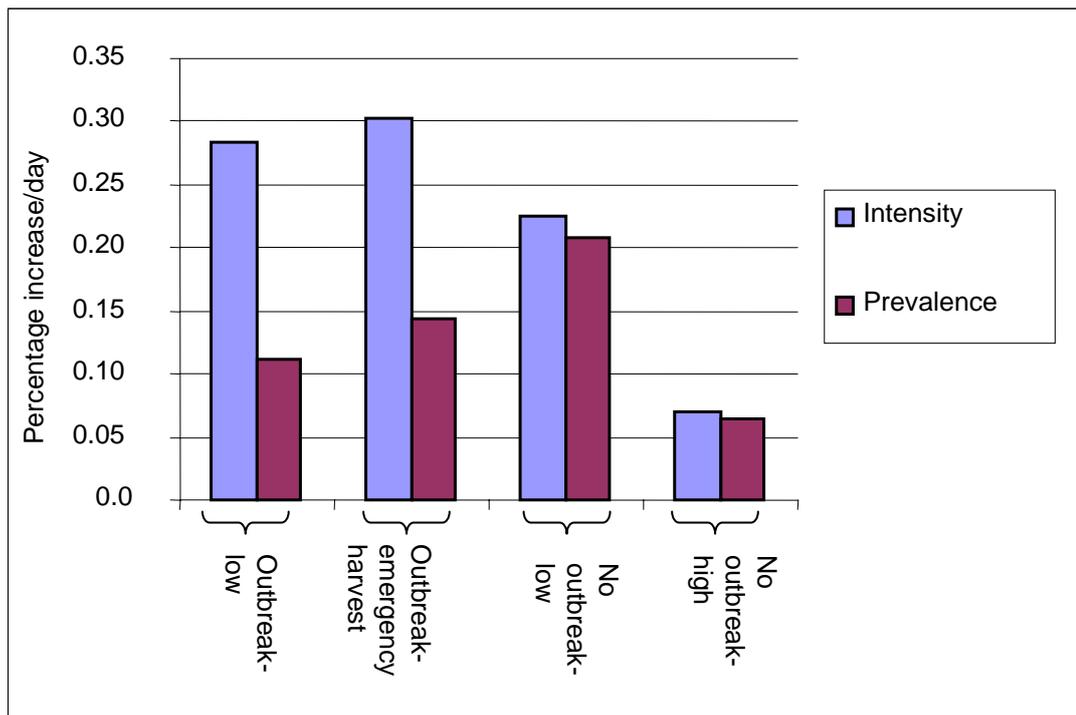


Figure 4.2. Daily percentage increase in prevalence and intensity of the farms over the production period. Note: Outbreak – low = GAV related outbreak – low production, Outbreak – emergency harvest = GAV related outbreak – emergency harvest, No outbreak – low = no GAV related outbreak – low production, No outbreak – high = no GAV related outbreak – high production

The daily increase in prevalence and intensity resulted in an overall uneven increase in both the prevalence and intensity within the groups of ponds (Figure 4.3 and 4.4). These results show that GAV had an association with production, and the ponds having GAV related outbreaks or the ponds with no obvious GAV related outbreak but low production, had the highest increases in prevalence and intensity, of GAV.

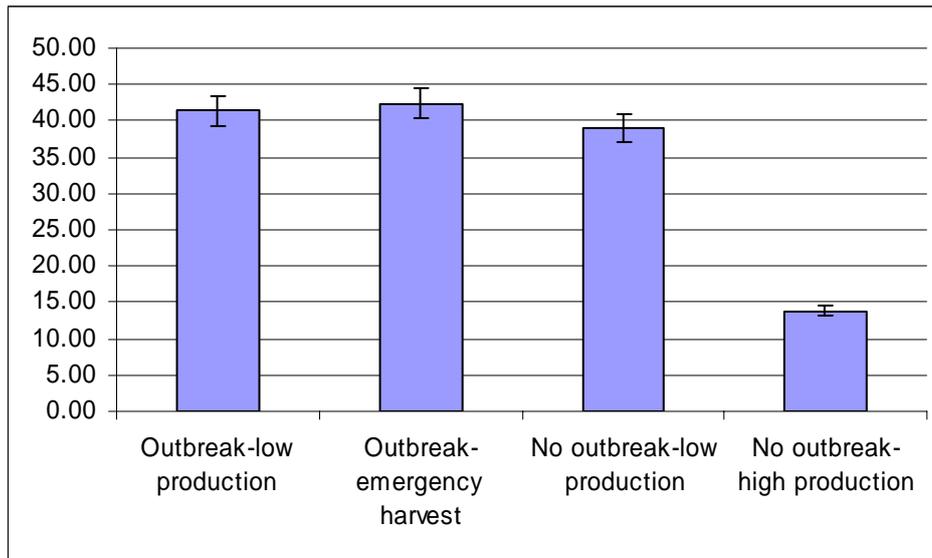


Figure 4.3. Percentage increase of GAV intensity over the production period. Note: Outbreak – low = GAV related outbreak – low production, Outbreak – emergency harvest = GAV related outbreak – emergency harvest, No outbreak – low = no GAV related outbreak – low production, No outbreak – high = no GAV related outbreak – high production

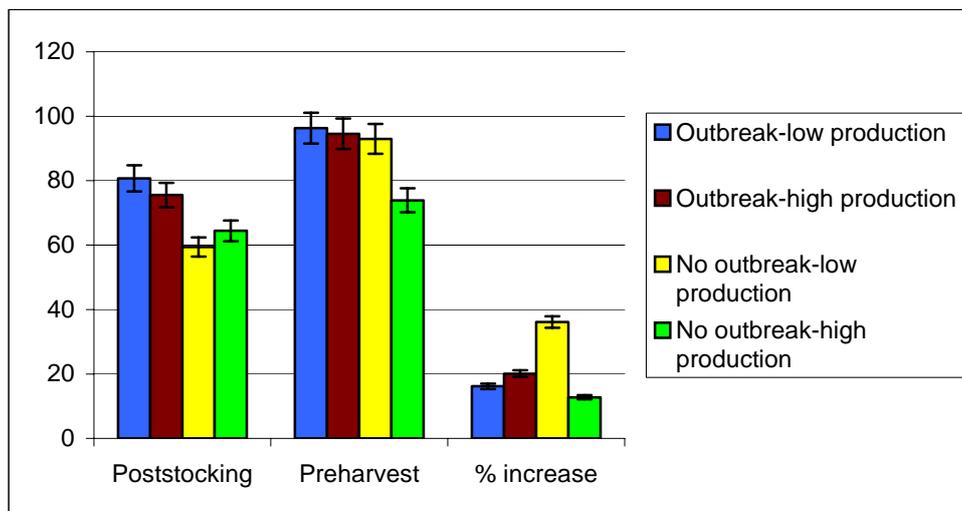


Figure 4.4 Prevalence of GAV from the ponds at poststocking, preharvest and the percentage increase over the production period. Note: Outbreak – low = GAV related outbreak – low production, Outbreak – emergency harvest = GAV related outbreak – emergency harvest, No outbreak – low = no GAV related outbreak – low production, No outbreak – high = no GAV related outbreak – high production

There was a significant difference in prevalence between pond groups at post stocking from a sample size of 900 prawns ($P = <0.001$, $DF = 3, 41$, $F = 8.627$).

However, there was no significant difference in prevalence between ponds that had GAV related outbreak – low production and ponds with GAV related outbreaks –

emergency harvest ($P = 0.250$) (Table 4.3). Also, there was no significant difference between the ponds that had no GAV related outbreak – low production and the ponds that had no GAV related outbreak – high production (Table 4.3). This indicates that the prevalence of GAV in the postlarvae from the hatchery can influence production while there are still factors occurring on the farm that can also influence the prevalence of GAV during the grow-out period, both having an association with decreased production. Unlike GAV prevalence at poststocking, there was no significant difference between ponds at stocking with GAV intensity ($P = 0.369$, $DF = 3, 819$, $F = 7.229$) (Table 4.3). Therefore, the different levels of intensity later in the crop were due to factors occurring on the farm.

Table 4.3. Significant differences in prevalence (lower triangle) and intensity (upper triangle) of GAV between ponds poststocking. Note: Outbreak – low = GAV related outbreak – low production, Outbreak – harvest = GAV related outbreak – emergency harvest, No outbreak – low = no GAV related outbreak – low production, No outbreak – high = no GAV related outbreak – high production

		Outbreak - low	Outbreak - harvest	No outbreak - low	No outbreak - high	
Prevalence	Outbreak - low		0.257	0.132	0.186	Intensity
	Outbreak - harvest	0.25		0.671	0.832	
	No outbreak - low	<0.001	0.003		0.83	
	No outbreak - high	0.001	0.032	0.342		

At preharvest there was an overall significant difference in prevalence between the groups of ponds ($P = <0.001$, $DF = 3, 41$, $F = 18.952$). The ponds with no GAV-related outbreak – high production were significantly lower in GAV prevalence than the other groups of ponds ($P = <0.001$, $DF = 3, 819$, $F = 62.605$) (Table 4.4, Figure 4.4, Figure 4.5). However, not all groups were significantly different to each other with respect to intensity of GAV. For intensity, the ponds with GAV related outbreak – low production were significantly different to the ponds with no GAV related outbreak – low production ($P = 0.003$) and the ponds with no GAV related outbreak – high production were significantly different to all the other three groups (Table 4.4, Figure 4.5).

Table 4.4. Significant differences in prevalence (lower triangle) and intensity (upper triangle) of GAV between ponds preharvest. Note: Outbreak – low = GAV related outbreak – low production, Outbreak – harvest = GAV related outbreak – emergency harvest, No outbreak – low = no GAV related outbreak – low production, No outbreak – high = no GAV related outbreak – high production

		Outbreak - low	Outbreak - harvest	No outbreak - low	No outbreak - high	
Outbreak - low	Prevalence		0.083	0.003	<0.001	Intensity
Outbreak - harvest		0.447		0.157	<0.001	
No outbreak - low		0.246	0.673		<0.012	
No outbreak - high		<0.001	<0.001	<0.001		

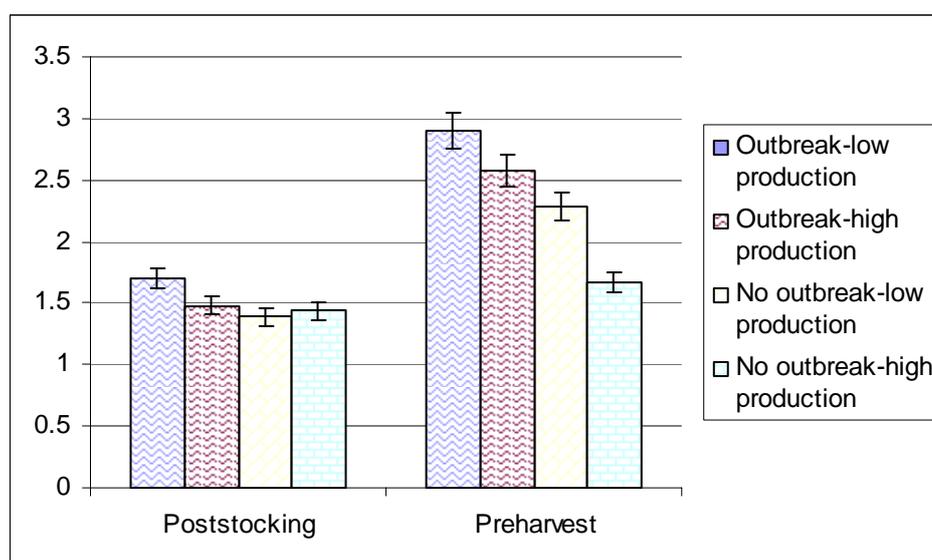


Figure 4.5. GAV intensity in ponds at poststocking and at preharvest. Note: Outbreak – low = GAV related outbreak – low production, Outbreak – emergency harvest = GAV related outbreak – emergency harvest, No outbreak – low = no GAV related outbreak – low production, No outbreak – high = no GAV related outbreak – high production

There was a significant difference in survival between the different groups of ponds at harvest ($P = <001$, $DF = 3, 44$, $F = 9.149$). However, there was no significant difference between the ponds that had GAV-related outbreak – low production and ponds with no GAV related outbreak – low production ($P = 0.212$) (Table 4.5). Also, there was no significant difference in survival between the ponds that had GAV-related outbreak – emergency harvest and the ponds that had no GAV-related outbreaks – high production ($P = 0.977$) (Table 4.5). This is because the act of emergency harvesting captured prawns before they succumbed to the GAV-related outbreak.

Table 4.5. Significant differences in survival in ponds that had GAV-related outbreaks-low production, GAV-related outbreaks-emergency harvest, no GAV-related outbreaks-low production and no GAV-related outbreaks-high production. Note: Outbreak – low = GAV related outbreak – low production, Outbreak – harvest = GAV related outbreak – emergency harvest, No outbreak – low = no GAV related outbreak – low production, No outbreak – high = no GAV related outbreak – high production

	Outbreak - low	Outbreak - harvest	No outbreak - low	No outbreak - high
Outbreak - low	N/A			
Outbreak - harvest	<0.001	N/A		
No outbreak - low	0.212	0.009	N/A	
No outbreak - high	<0.001	0.977	0.010	N/A

4.4 Discussion

The method of using 1-step and 2-step PCR as a tool to determine the prevalence and semi-quantitative load of virus that we used in this study is not unique.

Kiatpathomchai *et al.*, (2001) used a single tube, non-stop, semi-nested PCR technique to determine the prevalence and severity of disease of white spot syndrome virus (WSSV) infection in *P. monodon*. Depending on the severity of infection, different numbers of PCR products were amplified, indicating whether the prawn was either heavily infected, moderately infected or lightly infected. Similarly, Thakur *et al.*, (2002) used PCR to study the prevalence of WSSV in postlarvae (PL). They reported that samples which were 1-step PCR positive had at least 10^3 to 10^4 times more viral DNA than samples that were only positive by 2-step PCR and subsequently graded the PL batches and individual PL as heavily infected (1-step positive) and lightly infected (2-step positive). In our study we used a similar method to determine the semi-quantitative load of GAV infection in *P. monodon*. However in this study we visually graded the PCR product within the 1-step PCR or 2-step PCR. To do this we validated that the intensity of the PCR product from either the 1st step PCR or 2-step PCR on the agarose gel was semi-proportional to the relative amount of viral RNA present in the initial total RNA sample. It can be seen that the concentration of GAV viral RNA genome is semi-proportional to the intensity of the PCR amplicon (Figure 4.1). We are confident that this grading represents a true clinical PCR as we used crustacean RNA rather than PCR grade water to dilute ten-fold the RNA samples, as genomic RNA may affect the PCR. From this result we

determined that our grading system for both the outer and inner nest gives us a correct comparative semi-quantitative result of GAV loading in the prawn tissue. Withyachumnarnkul (1999) has previously demonstrated the use of PCR as a screening tool to reduce the likelihood of WSSV outbreaks in culture ponds of *P. monodon*. The paper from Withyachumnarnkul reports that the PLs were grossly healthy at the time of stocking and were tested for WSSV by 1-step PCR at 2 week intervals. It is reported that ponds that were stocked with WSSV positive PLs showed signs of white spot disease at an average of 40 days after stocking and that they had a 95.3% chance of occurring an emergency harvest compared to the ponds that remained WSSV PCR negative throughout the study which had a 1.9% chance of emergency harvest. It was also reported that, after a pond which was stocked as WSSV PCR negative was detected as being positive for WSSV, the outbreak time was approximately 45 days.

In this paper we demonstrated that GAV is associated with disease in *P. monodon* which affects survival and overall production in Australian farms. It was demonstrated that GAV increases in prevalence and in semi-quantitative viral load (intensity) over the production period. Both the prevalence and the semi-quantitative load have independent effects on production. The ponds that had the lowest increases in prevalence and semi-quantitative load of GAV had the highest production and thus no GAV-related outbreaks. It appears that ponds that had both a comparatively high prevalence of GAV at stocking and a relatively high increase in semi-quantitative load of GAV suffered a GAV-related outbreak. Ponds that were not diagnosed as suffering from a obvious GAV-related outbreak but had low production still had an association with GAV. There was an increase in prevalence and semi-quantitative GAV load over the production period, compared to the ponds that suffered the least from GAV-related disease (ponds with no GAV-related outbreak – high production). The ponds that suffered GAV-related outbreaks had a similar increase in semi-quantitative load as the ponds that suffered no GAV-related outbreak – low production, however they had a significantly higher prevalence at stocking. This possibly caused the obvious GAV-related outbreak. In the ponds classified as no GAV related outbreak – low production, prevalence and semi-quantitative load of GAV were comparatively low at stocking but had a subsequent high increase during the production period. This steady increase during the season may have resulted in

unnoticed, chronic mortalities and thus not being classified as suffering a disease outbreak. The association of GAV with disease is also supported with the percentage increase of GAV during the production period. The ponds with no GAV-related outbreaks –high production had the lowest increase in both prevalence and semi-quantitative load over the production period (12.79% and 13.77% respectively) with a daily increase of both prevalence and semi-quantitative load of 0.07%. The daily increase of GAV is not a true representative of the actual day to day increase but an average across the two sampling times used. However, it demonstrates the comparatively low increase in GAV of the ponds with no GAV-related outbreak-high production when compared to the ponds which were either classified as having a GAV-related outbreak or no GAV-related outbreak-low production (0.28 %, 0.30 % and 0.23 % respectively). The limit of using the daily percentage increase is that it does not determine if there were peaks in GAV increase or if it was a gradual increase over the production period. In future research, increased sampling times would clarify this point.

It appears that the GAV status of PL that are stocked into the ponds can affect production. The ponds which had significantly higher prevalence of GAV at stocking suffered GAV-related outbreaks. However, the conditions on the farm also affect prevalence as seen by the uneven increase in prevalence over the production period. This can especially be seen in the ponds classified as no GAV-related outbreak – low production, where these ponds had the lowest initial prevalence but the highest increase in prevalence over the production period. Unlike prevalence, there was no significant difference in the semi-quantitative load of GAV post-stocking, indicating that the increase in semi-quantitative load of GAV is due only to factors occurring on the farm.

In conclusion, there was an obvious association between the survival of stock and GAV which also directly affected production. The ponds that had GAV-related outbreaks had the highest prevalence and semi-quantitative load of GAV at one month post-stocking and at pre-harvest. The ponds which were classified as having no GAV-related outbreak – low production had the lowest initial prevalence and semi-quantitative load of GAV at one month post-stocking but had the highest increase in prevalence of GAV and a comparatively high semi-quantitative load of

GAV compared to the ponds with no GAV-related outbreaks – high production. This high increase during the production period was possibly causing undetected low levels of mortality throughout production. However, this study does not demonstrate that GAV directly or indirectly causes disease on Australian prawn farms since no other viruses were screened for prevalence or semi-quantitative load. To determine if GAV exhibits a causal relationship with disease and mortality in *P. monodon*, either a viable prawn cell line would need to be available or virus-free *P. monodon* would be required to be injected with purified, viable GAV. Until either of these two methods are available, this current study demonstrates that the prevalence and loading of GAV has a strong inverse association with survival of *P. monodon* on Australian prawn farms or at the very least is a strong indicator of the likelihood of a GAV-related disease outbreak or poor prawn survival.

This study has also demonstrated that farm management can have a large influence on a GAV-related outbreak and on the loss of production. It can be seen that by using RT-nPCR as a screening tool, if juveniles within one month of stocking have prevalence above 75%, there is a 95% chance of a GAV-related outbreak occurring within the production period. Likewise, if during the production period the prevalence of GAV is above 90% and semi-quantitative load is above 2, then there is a 95% chance that production will reduce if no action is taken. However, if screening for GAV using PCR is not viable and a GAV-related outbreak occurs (as defined above) then it is advisable to emergency harvest the pond. The ponds in this study that incurred a GAV-related outbreak and were emergency harvested had no significant difference in survival when compared to the ponds that had no GAV-related outbreaks-high production. The emergency harvested prawns would be naturally smaller, but implementation of this disease management strategy would minimise the economic loss in production.

CHAPTER 5

PRODUCTION OF POLYCLONAL AND MONOCLONAL ANTIBODIES AGAINST GILL-ASSOCIATED VIRUS AND THE DEVELOPMENT OF AN ELISA

5.1 Introduction

Gill-associated virus (GAV) of *Penaeus monodon* (black tiger prawn) and by inference, yellow head virus (YHV) is the type species of the *genus Okavirus* in the *Roniviridae* of the Nidovirales (Mayo 2002) and has been associated with mortalities in farmed *P. monodon* (Chantanachookin *et al.* 1993, Spann *et al.* 1997). The two viruses are closely related, sharing 95.8% amino acid sequence and 85.1 % nucleotide identity (Cowley *et al.* 1999). The level of sequence divergence between YHV and GAV is much greater than individual isolates of GAV, which share 98.5 % identity at the nucleotide level, indicating that YHV and GAV represent distinct genetic lineages and that they are geographical topotypes (Cowley *et al.* 1999). Nadala *et al.* (1997) identified four structural proteins (170, 135, 67 and 22 kDa) from purified YHV, and by inference GAV and determined that the 135 kDa protein was glycosylated.

Attempts to prevent and control GAV/YHV associated disease have relied primarily on diagnosing and removing infected prawns from the farm population. Reverse transcription nested polymerase chain reaction (RT-nPCR) has been developed for the detection of both GAV and YHV (Wongteerasupaya *et al.*, 1997; Cowley *et al.*, 1999; Cowley *et al.*, 2004) and more recently, a real time PCR has been developed for the two viruses (Dhar *et al.*, 2002; Vega *et al.*, 2004). Despite these PCR techniques being highly sensitive for the detection of the viruses, there are practical limitations to their widespread application. These limitations include the requirement for specialised equipment, expensive molecular reagents and well-trained personnel. Consequently, these high costs result in the assay being non-viable for the majority of prawn farmers or small support laboratories that screen for viruses.

Immunological techniques are widely used for the diagnosis of many viral diseases. They are highly sensitive and accurate for specific viruses and results can be quickly obtained at a relatively low cost, making them ideal for on-farm screening of disease and pathogen load, when used correctly. Monoclonal antibodies (MAbs) to YHV have been previously reported (Sithigorngul *et al.*, 2000; Sithigorngul *et al.*, 2002; Soowannayan, Flegel, Sithigorngul, Slater, Hyatt, Cramerri, Wise, Crane, Cowley, McCulloch, and Walker 2003). However, the assay (Western blot, dot blot analysis and immunohistochemistry) could not be easily applied for field examinations. The present work describes the first development of polyclonal antibodies (PABs) and specific MAbs against GAV and their use in the detection of GAV by capture ELISA.

5.2 Materials and Methods

5.2.1 Purification of gill-associated virus

Methods used to purify GAV were adapted from Wongteerasupaya *et al.* (1995a) and Nadala *et al.* (1997). Fifty grams of *P. monodon* gill tissue was homogenised in 500 ml (1 in 10 w/v) of TNE buffer (.02 M Tris HCl, 0.2 M NaCl, 0.02 M EDTA pH 7.4). This was then centrifuged at $5000 \times g$ for 10 minutes in an Eppendorf centrifuge (A-4-44 rotor). The supernatant was then centrifuged again at $5000 \times g$ for 30 minutes in an Eppendorf centrifuge (A-4-44 rotor). The supernatant was then filter sterilised through a $0.45 \mu\text{m}$ filter and centrifuged at $100\,000 \times g$ for 1 hour in a Beckman ultracentrifuge (Ti 70.1 rotor). The resulting pellet was resuspended in TNE buffer and overlaid onto a 25 – 55 % sucrose gradient and centrifuged at $100\,000 \times g$ for 20 hours on a Beckman ultracentrifuge (SW40 rotor). After centrifugation, viral bands were visualised by top illumination, and removed using an 18-gauge needle and diluted 5 times in TNE before centrifugation at $100\,000 \times g$ for 1 hour. The pellet was then resuspended in 500 μl of phosphate buffered saline (PBS) (1.4 M NaCl, 0.014 M KH_2PO_4 , 0.08 M Na_2HPO_4) and stored at -20°C .

5.2.2 Polyacrylamide gel electrophoresis

One dimensional polyacrylamide gels of 7.5 cm length, using 0.75 mm spacers, with a 12% separating gel were prepared and run by the method of Pizzutto (1992), being

a modification of the method of Laemmli (1970). A mini Protean II apparatus (Bio-Rad) was used to electrophorese the gels. SDS running buffer (0.1 M $\text{NH}_2\text{C}(\text{CH}_2\text{OH})_3$, 1 M $\text{C}_2\text{H}_5\text{NO}_2$, 0.02 M $\text{C}_{12}\text{H}_{25}\text{O}_4\text{SNa}$, pH 8.3) was poured into the buffer tank containing the gels and 200 μl of sample was loaded into the two comb well. The sample consisted of 50% purified virus and 50% reducing buffer (0.06 M $\text{NH}_2\text{C}(\text{CH}_2\text{OH})_3$, pH 6.8, 10% glycerol, 5% CH_6OS , 0.05% (w/v) bromophenol blue). Five microliters of broad range SDS-PAGE molecular weight standards (Bio-Rad) were used as markers. Electrophoresis was performed at 200 V until the tracking dye left the bottom of the gel. Protein bands were either visualised by staining with Coomassie blue or transferred for Western blotting.

5.2.3 Production of polyclonal antibodies in chickens

A multiple emulsion of PBS with presumptive GAV protein subunits 170, 110 and 63 kDa emulsion (excised from the polyacrylamide gel) was prepared in a 1:1 ratio. When completely homogenised, one ml of the mixture was injected into the breast muscle of three semi-adult chickens.

On day 14 and 28 after the initial injection, a booster dose was administered using the same technique as the original injection. On day 25, three mls of blood was removed from each chicken via the wing vein. The blood was allowed to clot at room temperature for four hours. The serum was then removed and centrifuged at $1500 \times g$ for 10 min. The serum was then screened for PABs to the target antigen (method below) by indirect ELISA (section 5.2.11). On day 28, a further 10 ml of blood was removed from each chicken via the wing vein. The blood was then processed as before. The serum was subsequently stored in aliquots of 200 μl at -80°C .

5.2.4 Western blots

5.2.4.1 Protein transfer

Protein samples were run using the SDS-PAGE technique. The procedure for wet electrophoretic transfer was adapted from Towbin, Staehelin, and Gordon (1979). The gels were loaded into gel cassettes (Bio-Rad Laboratories) in direct contact with polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore, Australia). A

cooling module was inserted into each chamber. The chambers were then filled with transfer buffer and the complete unit was placed in a water bath at a constant 4°C. The blots were run at 50 V for 2.5 hours. They were then removed from the cassettes and dried overnight at room temperature.

5.2.4.2 Immunostaining Western blots

Once the membranes (blots) were thoroughly dry, they were incubated with either MAbs or PABs (primary antibody) for 1 hour. The membrane was washed twice for 5 minutes each time using fresh PBS. The secondary antibody, either rabbit anti-chicken IgG (H + L) - HRP conjugate (Bio-Rad Laboratory, USA, #170-6516) or goat anti-mouse IgG (H + L) - HRP conjugate (Jackson Immuno Research, USA) was added and blots were incubated for a further 1 hour, followed by washing three times for 5 min each time using fresh PBS. The colorimetric substrate 3,3',-diaminobenzidine tetrahydrochloride (DAB) (Sigma, Australia) was added and the colour development reaction was halted at the desired level by the addition of distilled water. All incubation steps were conducted at room temperature on an orbital shaker.

5.2.5 Visualisation of affinity purified protein from MAbs

The monoclonal antibody was purified using ImmunoPure IgM purification kit (Pierce) following the manufacturer's instructions. The antibody was then immobilized into an antibody support column using a primary immunoprecipitation kit (Pierce) following the manufacturer's instructions and the previously purified GAV samples were added to the column, bound to the immobilized antibody, purified via washing steps and eluted following the manufacturer's instructions. The eluted GAV protein was then analysed on SDS-PAGE after silver staining.

5.2.6 Fixation of tissue

Gill tissue was aseptically extracted from the prawn and was preserved in Davidson's fixative (4:3:2:1 (v/v) of H₂O: EtOH: CH₂O: COCH₃OH). After 24 hours, the tissue was transferred to 80% alcohol for subsequent histological preparations

5.2.7 Preparation of paraffin sections for immunostaining

Gill tissues from *P. monodon* and *P. merguensis* were dehydrated in graded ethanol, cleared in xylene and embedded in paraffin wax. Sections were cut at 5 µm and dried in an oven at 60°C for a minimum of 30 minutes. The dried sections were then taken to water through xylene and ethanol. All tissues were cut cross-sectionally.

5.2.8 Immunostaining of tissue sections

Prior to immunostaining, the sections were rehydrated through two changes of xylene for two minutes each, absolute ethanol for two minutes followed by two changes of absolute ethanol for one min each, and a water wash for one min. After the sections were hydrated, they were washed twice in PBS for five minutes each, then any endogenous peroxidase was blocked by covering the sections with 0.3% (w/v) hydrogen peroxide in methanol for 30 minutes. The sections were then washed once in PBS for 5 minutes. Sections were blocked with 10% normal goat serum to prevent non-specific binding and then washed once in PBS for 5 minutes. The monoclonal antibody, 3K5-1, was added to the sections at room temperature in a humid chamber for 1.5 hours and then washed three times in PBS. The sections were then covered in affinity purified goat anti-mouse IgG (H + L) - HRP conjugate diluted 1/120 in ELISA diluent (TroBio, Australia) and incubated as before for one hour. The slides were then washed as before and covered with DAB substrate solution for 10 minutes at room temperature then washed once with PBS. The sections were then counterstained in haematoxylin for 5 minutes, washed for one min in tap water, followed by blueing in Scott's Tap Water Substitute for 30 seconds. The sections were then washed for two min in water. The sections were then dehydrated through alcohols and xylene and mounted in D.P.X.

Controls for non-specific binding of the antibodies were done on sections run in parallel, using sections from *P. merguensis* gill tissue that had previously tested negative for GAV by RT-nPCR.

5.2.9 Antigen preparation for development of indirect and capture ELISA

Antigen was prepared by homogenising 0.3 g of *P. monodon* gill tissue in 200 µl carbonate buffer (0.04 M NaHCO₃, 0.01 M Na₂CO₃, pH 9.6) and centrifuged at 5000 × *g* for 10 minutes in an Eppendorf centrifuge (A-4-44 rotor). The supernatant was then used to determine the optimum dilution by checker board titration. As a negative control, gill tissue from *P. merguensis* that tested negative for GAV by RT-nPCR was used.

5.2.10 Optimisation of indirect ELISA

An indirect ELISA was developed to screen for PABs to the specific antigens and to determine the optimal PABs titre. Checkerboard titrations were carried out to determine optimal dilutions of the antigen and PABs. The optimal dilution was defined as the reciprocal of the highest dilution that gave a optical density of 1.0 in one hour. The antigen was serially diluted one in two (v/v) in carbonate buffer across the plate starting at a 1 in 10 dilution and finishing at a 1 in 19520 dilution. The quantity of antigen and PABs in the initial ELISA plate wells was 100 µl. This was then dried overnight at 37°C prior to adding the PABs. The PABs were serially diluted down the plate starting at a 1 in 10 dilution and finishing at 1 in 1220. The ELISA plates for the indirect ELISA were IWAKI cell culture plates (Crown Scientific, Australia) flat bottomed, 96 well, polystyrene coated. Results were read with an ELISA reader (Labsystems, Multiscan EX) using dual optical density (O.D.) of 414 and 492 nm.

The incubation steps for the ELISA were one-hour durations at room temperature. After the antigen had been coated onto the plate and dried overnight, 150 µl of post-coating buffer (TropBio, Townsville) was added and incubated, 100 µl of the PABs was added to the ELISA plate wells and further incubated. Rabbit anti-chicken IgG (H + L) - HRP conjugate (secondary antibody) (Bio-Rad Laboratory, USA, #170-6516) was then added in the same manner as the PABs. 2,2'-azino-di-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) (KPL Europe, Guilford UK) was added and incubated prior to determining the colour formation on the ELISA reader. Between each step, the ELISA plate was washed three times with ELISA wash buffer (TropBio, Australia).

5.2.11 Cell count

The concentration of viable cells was determined in a haemocytometer, using the trypan blue dye exclusion test. Equal volumes of cell suspension and 0.2% trypan blue solution were mixed, loaded into the counting chamber of the haemocytometer and were examined under a phase contrast optics, inverted microscope. The number of viable cells per ml of the medium was determined by counting the unstained cells in the corners of the haemocytometer and applying the following formula:

$$\text{Concentration of viable cells (cells/ml)} = \frac{\text{Number of viable cells counted}}{\text{Number of corners counted}} \times 2 \times 10^4$$

5.2.12 Production of monoclonal antibodies in Balb/c mice

5.2.12.1 Immunisation schedule

Seven male Balb/c mice approximately 9 weeks old were injected intraperitoneal (i.p) with a 300 µl dose containing an emulsion of either a high molecular weight, mid molecular weight or low molecular weight protein sub-units from PAGE gels of GAV and PBS. The mice were boosted at day 14 and day 28 after the initial injection.

One week after the final injection, tail bleeds were carried out on each mouse. Blood (10 µl) was collected onto filter paper discs (TropBio, Townsville) and each disc was placed into 500 µl PBS. Antibody titres were assessed by capture ELISA. Seven days before the planned fusion, the mouse was boosted with the usual 300 µl of the specific protein band, plus 50 µl of undiluted purified GAV into the tail vein.

5.2.12.2 Culture of myeloma cells

The myeloma cells (Sp2/0) were cultured in flat-bottomed 25 cm² polystyrene flasks in a CSL MDM medium (TropBio, Townsville) and supplemented with 10% bovine donor serum (BDS) (Starrate Pty Ltd, Wagga Wagga, Australia). The cultures were incubated at 37°C in a humid, 5% CO₂-enriched atmosphere. The day prior to fusion,

the myeloma cells were counted and divided into 75 cm² flasks with 50 ml CSL + 20% foetal bovine serum (FBS) (Starrate Pty Ltd, Wagga Wagga, Australia) + 0.5 ml of 1 × oxaloacetate-pyruvate-insulin media supplement (OPI) to provide a monolayer of approximately 80% confluence with 90% viability on the day of fusion.

5.2.12.3 Optimisation of capture ELISA

The capture ELISA (Figure 5.1) was used to screen mouse antibody production and to screen hybridoma cells. Unless otherwise stated, all steps were performed using 50 µl of each reagent with a reaction period of 1 hour at room temperature. Between each step, the ELISA plate (96 U bottom plates, Starstedt, Australia) was washed 5 times with washing buffer (TropBio, Townsville, Australia).

PAbs were diluted at 1 in 80 and added to each well. This was then incubated overnight and washed once with TEN buffer (TropBio, Townsville, Australia). The plates were blocked using 150 µl post-coating buffer (TropBio, Townsville, Australia) for 1 hour. Antigen was added to the ELISA plate wells and incubated. Either cell culture fluid or mouse serum was then added to the plates and incubated. ABTS was added and incubated prior to determining the intensity of colour formation using an ELISA plate reader (Labsystems, Multiscan EX).

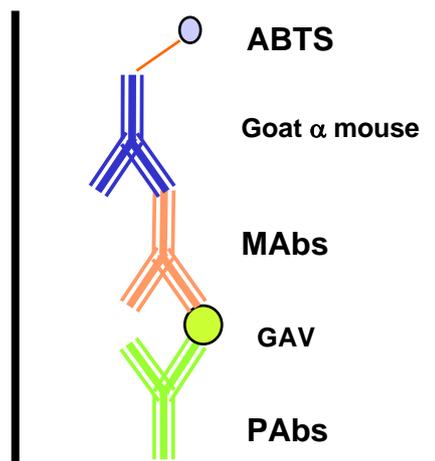


Figure 5.1. A diagrammatic representation of the capture ELISA used in this study to detect GAV

5.2.12.4 Screening for GAV using capture ELISA

PABs were diluted at 1 in 80 and 50 µl of this was added to each well. This was then incubated overnight and then washed once with TEN buffer (TropBio, Townsville, Australia). The plates were then blocked using 150 µl post-coating buffer (TropBio, Townsville, Australia) for 2 hours. A single gill filament from the posterior end of the prawn gills was homogenised and diluted to 1:128 in carbonate buffer and centrifuged at 12000 x g for 10 minutes. The supernatant was added to the ELISA plate wells and incubated. Cell culture fluid was added to the plates and incubated. ABTS was added and incubated prior to determining the colour formation with the ELISA reader (Labsystems, Multiscan EX).

5.2.12.5 Preparation of spleen cells

The method used in the preparation of spleen cells was adapted from Zola (1995). Immediately prior to fusion, the mouse was killed with CO₂ and soaked in 70% ethanol. The spleen was aseptically removed from the peritoneum and excess fat was carefully removed. The spleen was then transferred into a vial containing 10 ml of CSL MDM at 37°C. The dead mouse was removed and the area was resterilised. The spleen was transferred into a Petri dish containing approximately 10 ml of CSL MDM at 37°C. The splenocytes were purged by repeatedly injecting CSL MDM media from the Petri dish into the spleen using a three ml syringe with a 23-gauge needle. After most of the splenocytes were removed, the spleen capsule was massaged with a pair of sterile spreaders to remove any remaining cells. Clumps of cells were separated by gently aspirating in and out of the 23-gauge needle. The spleen capsule and other visible lumps were removed. The media containing splenocytes in the Petri dish was transferred to a 50 ml centrifuge tube and the Petri dish was washed with approximately 15 ml of CSL MDM at 37°C and this was also added to the 50 ml centrifuge tube. One ml of BDS was deposited into the bottom of the 50 ml centrifuge tube to underlay the cell suspension. This was allowed to stand for five minutes so any large lumps could come out of suspension. The cells above the BDS were transferred into another 50 ml centrifuge tube. The cells were counted in a haemocytometer before centrifuging at 200 × g for five minutes.

5.2.12.6 Fusion protocol

Ten grams of polyethylene glycol (PEG) (MW 3000 – 3700) (Sigma) was autoclaved at 121°C for 30 minutes. While the PEG was liquid, 14 ml of 15% dimethyl sulfoxide (DMSO) in PBS was added and mixed. This was divided into one ml aliquots and stored at -20°C. Prior to use, it was microwaved on HIGH for one minute and held at 37°C for use.

Prior to fusion, the splenocytes were resuspended and washed in approximately four ml of CSL MDM. Myeloma cells were added at a ratio of spleen cells to myeloma cells of 10:1 into a 50 ml centrifuge tube. This mixture of cells was pelleted at $300 \times g$ for 5 minutes. After centrifugation, the medium was decanted and the cells were resuspended and mixed with one ml of warm PEG solution for one minute to allow fusion to occur. Three mls of 37°C CSL MDM was added drop-wise over a ten minute period and a further seven mls of 37 °C CSL MDM was added drop-wise over another ten minute period. During this time the cells were continually mixed. The cells were pelleted at $200 \times g$ for five min and resuspended in 100 ml of CSL MDM + 20% FBS + 2% hypoxanthine-aminopterin-thymidine (HAT) + 1% OPI media. The mixture was plated out at $100 \mu\text{l well}^{-1}$ into 96-well cell culture plates (Nunc, Denmark) and incubated at 37°C in a humid 5% CO₂ incubator. On day seven, the wells were examined for colony development, with an inverted phase contrast microscope, and a further 100 μl of fresh medium was added carefully into the wells without disturbing the cells. The medium was the same as before except HAT was replaced by hypoxanthine-thymidine (HT).

5.2.12.7 Screening for antibody production

Hybridoma colonies were screened for antibody production to the specific antigen on day 14. Using a capture ELISA for each screening, 100 μl of medium was transferred from a well in the cell culture plate into a well in the ELISA plate. The cell culture plates were topped up with 100 μl of fresh medium/well.

5.2.12.8 Cloning of monoclones

Single colonies that were reactive to the specific antigen were transferred into a 24-well cell culture plate containing CSL MDM, 20% FBS, 2% HT when an 80% confluence was reached.

Cloning was started from 24-well plates, with the remaining cells grown up in 25 cm² flasks for cryopreservation. After cell counts were determined, cells at a calculated density of two cells per well were added to 96-well cell culture plates. This resulted in an increased number of wells with growth and on average, resulted in 10 wells per plate containing single colonies. The resulting colonies were screened by capture ELISA for the production of MAbs. These hybridomas were recloned until all wells containing single colonies tested positive against the specific antigen.

5.2.13 Cryopreservation of cell lines

5.2.13.1 Freezing cultured cells

Prior to cryopreservation, cells were observed to ensure that they were visually healthy and were rapidly dividing. A confluence of 80% was obtained prior to cells being aspirated off the surface of the flask and frozen. The cell suspension was centrifuged at $300 \times g$ for 5 minutes. The cell pellet was resuspended in a one ml solution of 70% CSL - MDM, 20% BDS and 10% DMSO if it was from a 25 cm² flask or into a 3 ml solution if it was from a 75 cm² flask. One ml aliquots were transferred into 1 ml cryopreservation tubes (NUNC, Denmark) and placed in a storage cane which was then inserted into a cardboard sleeve and placed into a -80°C freezer for 24 hours before being appropriately labelled and placed into liquid nitrogen.

5.2.13.2 Thawing cells from liquid nitrogen storage

Frozen cells were thawed rapidly by semi-immersion of the cryopreservation tube in a 37°C water bath. The tube was centrifuged at $900 \times g$ for 5 minutes. The tube was surface sterilised with 70% ethanol, the supernatant was removed, the cell pellet was

resuspended in 1 ml of 37 °C culture medium and then transferred to a 25 cm² cell culture flask with 10 ml of 37 °C culture medium.

5.2.14 Antibody isotyping

Isotyping of the monoclonal antibodies was performed using SIGMA ImmunoType mouse monoclonal antibody isotyping kit (ISO-1) (Sigma-Aldrich, St. Louis USA), following the manufacturer's instructions.

5.2.15 RNA isolation

Total RNA was extracted following a modified method of Cowley *et al.* (2000). Briefly, a single gill filament was removed from 20 prawns for each species. These were then homogenised in 1000 µl of TRIzol (Life Technologies) and total RNA was isolated following the manufacturer's instructions.

5.2.16 Reverse transcription (RT) – PCR

The method to reverse transcribe the RNA to cDNA was adapted from Cowley *et al.* (2000).

5.2.17 PCR primers

The primers used in this study for the detection of GAV were developed by Cowley *et al.* (2000). Briefly, the outer nest primers were GAV-5 (5'-AAT TTT GCC ATC CTC GTC AC-3') and GAV-6 (5'-TGG ATG TTG TGT GTT CTC AAC-3') amplified a 618 base pair (bp) region of the genome. The inner nested primers were GAV-1 (5'-ATC CAT ACT ACT CTA AAC TTC C-3') and GAV-2 (5'-GAA TTT CTC GAA CAA CAG ACG-3') amplified a 317 bp region of the genome.

5.2.18 Animal Ethics

Ethics for this project was granted through James Cook University ethics review committee. Ethics approval numbers were: A832 and A947.

5.3 Results

5.3.1 Development of an ELISA for GAV

The dilution factor of 1/128 was chosen to be the optimal dilution for the optical density of the antigen (Fig 5.2). This dilution was used in the capture ELISA for screening monoclonal antibodies.

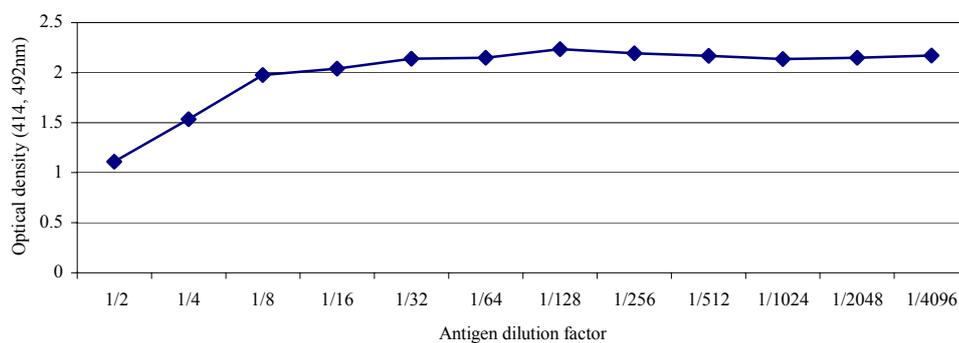


Figure 5.2. Titration of antigen for the optimisation of a capture ELISA

The dilution factor of 1/80 was chosen as the standard dilution of the optical density of the PABs (Fig 5.3). This dilution was chosen to give an optical density above 1.0 while having a relatively low background.

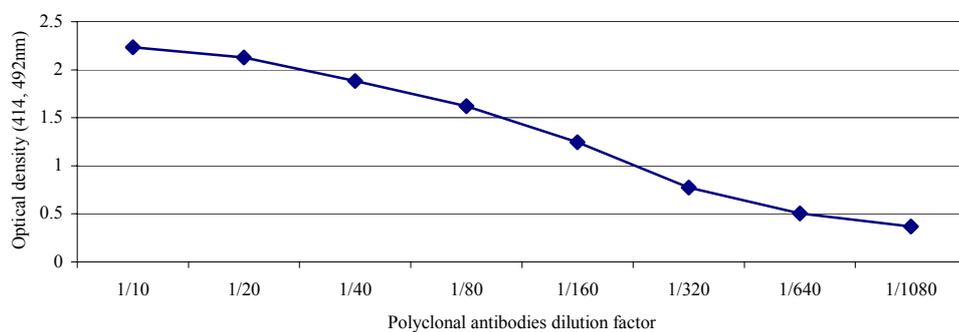


Figure 5.3. Titration of polyclonal antibodies for the optimisation of the capture ELISA.

Figure 5.4 shows the relative optical density of the developed monoclonal antibodies. The optical density is in response to the pre-optimised dilution factors of antigen and PAbs (Figure 5.2 and 5.3).

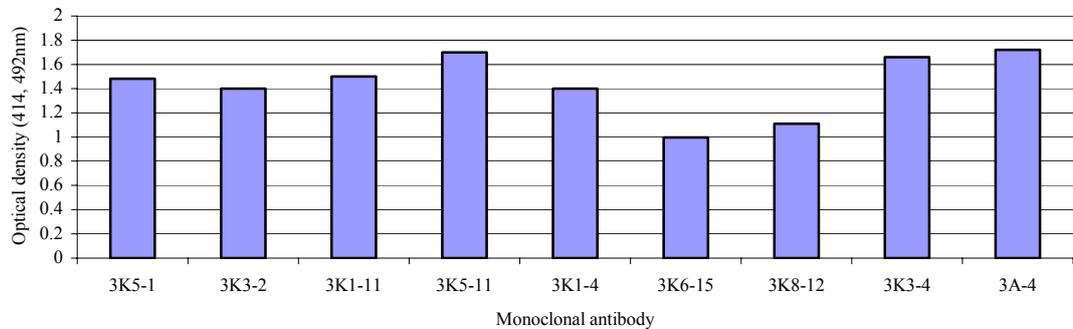


Figure 5.4. Measured optical density readings of the developed monoclonal antibodies to GAV in a capture ELISA

5.3.2 Confirmation that the developed MAb and PAb antibodies are specific for GAV

The 3K5-11 MAb were specific to a single protein band of GAV (20 kDa) (Figure 5.5) The PAb detected the three other protein bands that are presumptive GAV (170, 110 and 63 kDa) (Figure 5.6).



Figure 5.5. Western blot using 3K5-11 MAb against purified GAV detecting a single protein band of 20 kDa

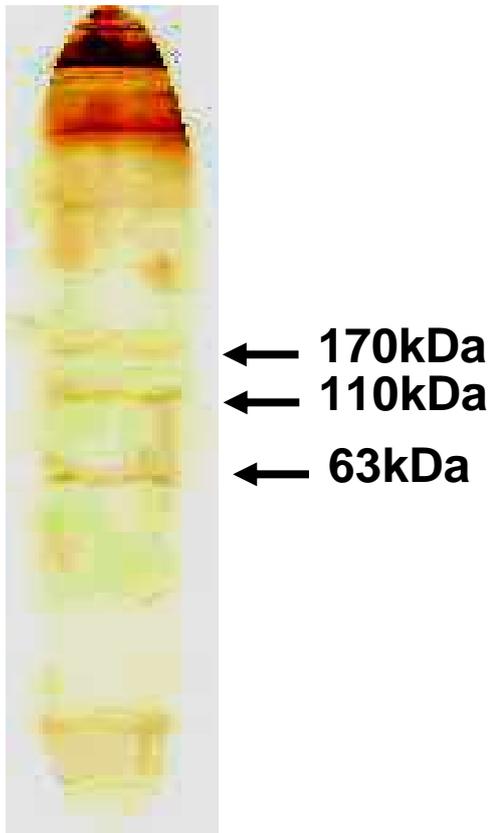


Figure 5.6. Western blot of PABs against purified GAV showing three protein bands of 170, 110 and 63 kDa respectively

The MAb purified a single protein of approximately 20 kDa (Figure 5.7). This is the correct size for the smallest protein of GAV.

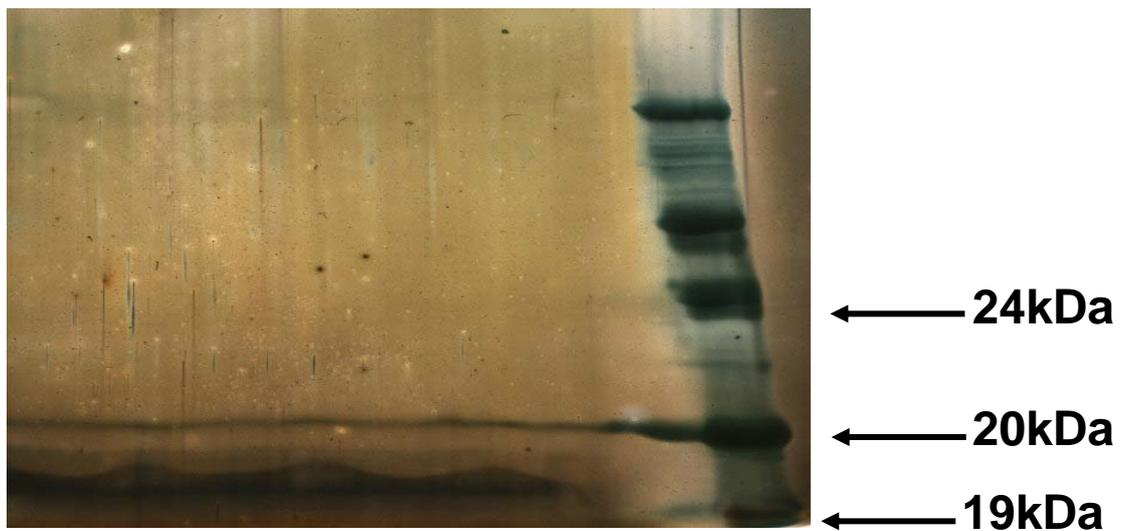


Figure 5.7. Affinity purified GAV protein eluted from the capture 3K5-11 MAb, visualised by PAGE and stained with silver stain

The MAb appeared specific to GAV and did not bind with prawn protein (Figures 5.8 and 5.9). It can be seen that MAbs are bound in the gills of the *P. monodon* (Figure 5.8) which has previously been shown to be infected with GAV via RT-nPCR while no MAb bound to the gills of the *P. merguensis* (Figure 5.9) which had previously been shown to be negative for GAV via RT-nPCR.

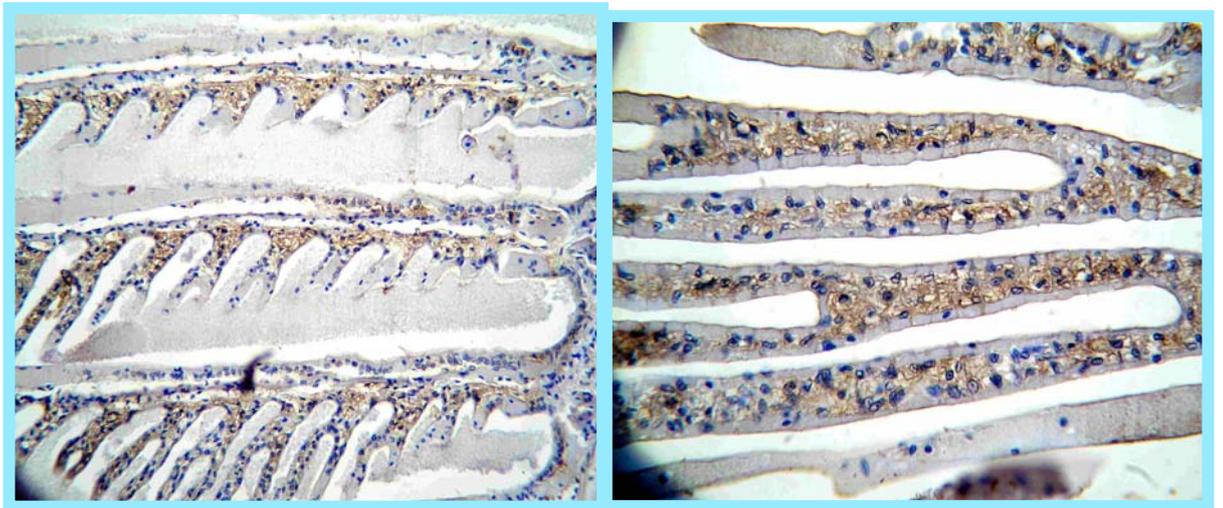


Figure 5.8. Immunostaining gills of *P. monodon* using 3K5-11 MAb raised against GAV to demonstrate potential specificity towards GAV

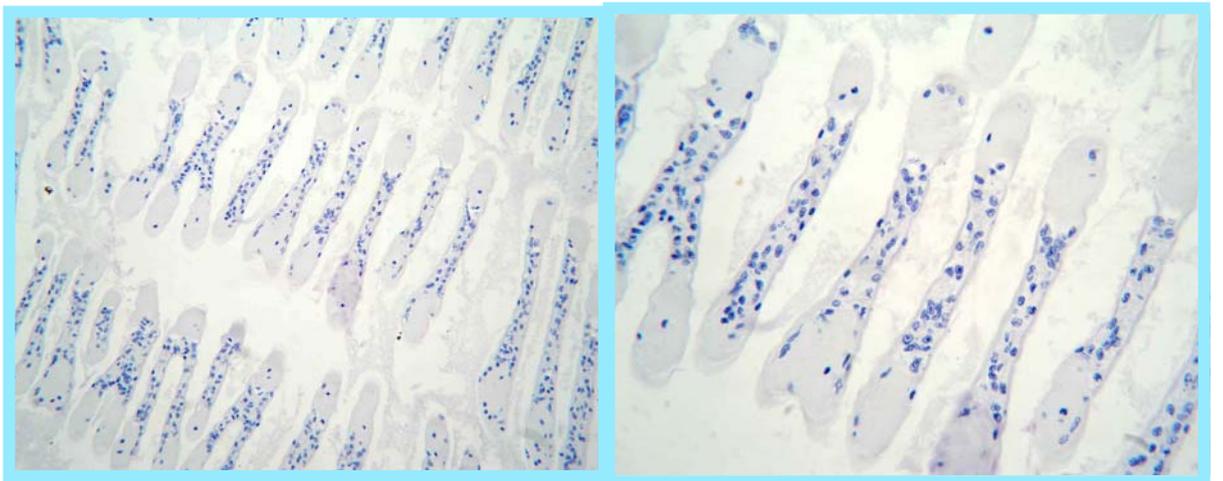


Figure 5.9. A lack of immunostaining with gills of *P. merguensis* using 3K5-11 MAb raised against GAV demonstrating the antibodies are not binding to prawn gills

All monoclones produced were of the IgM isotype.

The capture ELISA using MAb 3K5-11 was used to detect GAV presence and loading in 20 *P. monodon* (Figure 5.10) which had previously been shown to be infected with GAV by RT-nPCR (results not shown). In this experiment the control was from *P. merguensis* gill that had previously been shown to be GAV negative by RT-nPCR (results not shown). Prawns 1, 2 and 4 were highly infected with GAV, while prawns 3, 5, 8, 9, 10, 13, 17, 18 and 20 were moderately infected with GAV and prawns 6, 7, 11, 12, 14, 15, 16 and 19 had a low infection of GAV (Figure 5.10).

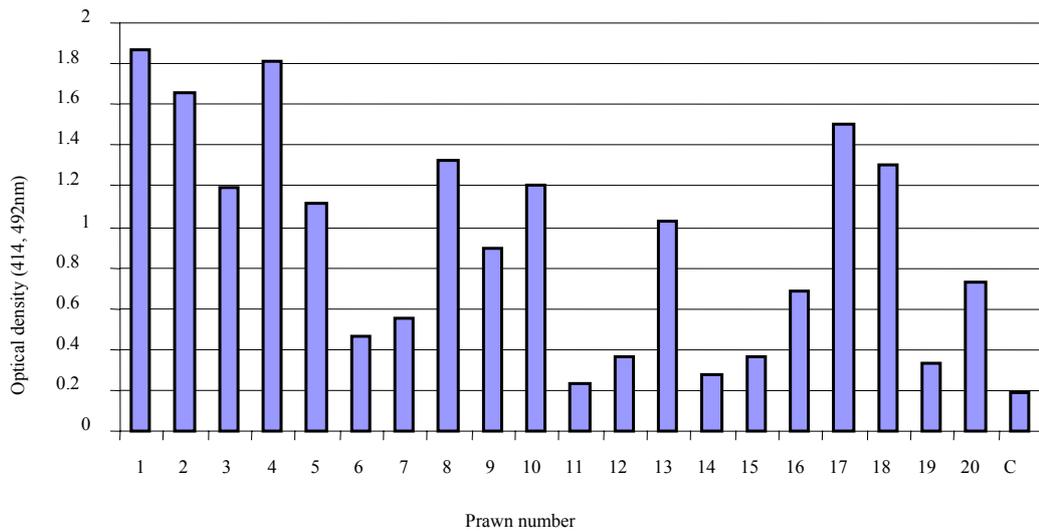


Figure 5.10. Capture ELISA using a single prawn gill filament to obtain the antigen. The filament was homogenised and diluted to 1:128. Note: prawns 1 – 20 are *Penaeus monodon*. (C) – Control = *Penaeus merguensis*

5.4 Discussion

This chapter reported the development of PAbs and MAbs that can be used for the detection of GAV in a capture ELISA. Nadala *et al.* (1997) wrote that GAV consists of four protein subunits, being 170, 135, 67 and 22 kDa respectively. However, Wang and Chang (2000) subsequently reported that there are only three major proteins in YHV, and by inference GAV proteins (110, 63 and 20 kDa) and suggested that the larger protein (170 kDa) may have been of prawn cellular origin. Jitrapakdee, Unajak, Sittidilokratna, Hodgson, Cowley, Walker, Panyim, and Boonsaeng. (2003) demonstrated that the larger two of these three proteins are glycosylated. Alternatively, the method used by Wang and Chang (2000) to purify the virus and to reduce it into subunits may have caused cleaving of the 170 kDa

protein into the reported 110 kDa and 63 kDa respectively. The antibodies herein were developed using the information from Nadala *et al.*,(1997), consequently the PAbs were shown to react to the approximate protein subunits 170, 110 and 63 kDa respectively using Western blot analysis. On the other hand the 3K5-11 MAb was shown to react to the 20 kDa protein subunit (approximately) using both a Western blot and an affinity purified GAV. These MAbs were then shown to be specific to GAV by immunohistochemistry using paraffin fixed gill tissue from *P. monodon* compared to *P. merguensis*. The MAbs gave positive staining in prawns that were positive for GAV with no staining in prawn tissue sections that were negative for GAV. These stained sections showed that the 3K5-11 MAb did not detect any protein in the prawn tissue and only detected presumptive proteins for GAV infected prawn tissue.

In 2005, Cowley *et al.* reported Mourilyan virus (MoV) as a new virus detected in *P. monodon* within Australia. Based on the morphological appearance, it was reported that this virus was Bunyavirus-like. If the classification is correct and along with the unknown prevalence of MoV, it is possible that the developed antibodies towards GAV are actually towards MoV due to the possible similar buoyant density (1.2 g/cm^3 in CsCl_2 for Bunyaviridae compared to 1.15 to 1.19 g/cm^3 in sucrose for GAV) (Calisher 1991; Brian, Hogue, and Kienzle 1995) and the similar protein profiles (200, 108-120, 29-41 and 19-25 kDa for Bunyaviridae, compared to 110, 63 and 20 kDa for GAV) (Calisher, 1991, Wang & Chang, 2000). Until the buoyancy and protein profiles have been determined for this newly discovered virus, this possibility cannot be discounted.

From the current results it is highly likely that the developed MAbs are specific for GAV and when used in an ELISA they are detecting GAV. The MAbs only detected the 20 kDa protein. It is hypothesised that this is due the screening method applied during the production of the MAbs. The screening method used was a capture ELISA using the PAbs which bound to the 170, 110 and 63 kDa proteins respectively (Figure 5.1) on the bottom part of the capture ELISA. Due to the PAbs attaching to the two larger proteins (three including presumptive prawn cell origin), there was only one protein left available for attachment of the second antibody. Therefore any MAbs that was not specific to the 20 kDa protein would not have been detected as it

would not be able to get a binding site for attachment. With the use of the developed antibodies, a capture ELISA is very efficient at detecting GAV in a prawn population (Figure 10) with *P. merguensis* gill tissue giving very low background in the ELISA. Even though the PABs may bind a protein subunit that is not from GAV, the specificity of the MAb ensures correct GAV detection.

The requirement of needing only a single gill filament results in this test being non-destructive to the prawn as a single filament can be removed easily, enabling it to be used for screening valuable broodstock. The low training and technology required enables this test to be used both on farms and in small support laboratories. Once the viral load of GAV necessary for a disease outbreak has been calibrated by ELISA, a sampling program can be introduced onto farms for the surveillance of GAV to prevent an outbreak.

CHAPTER 6

HAEMAGGLUTINATION AS A LOW COST DETECTION METHOD FOR GILL-ASSOCIATED VIRUS AND BY INFERENCE, YELLOW HEAD VIRUS IN *Penaeus monodon*

6.1 Introduction

Gill-associated virus (GAV) is a single-stranded, positive sense RNA virus that has been associated with massive loss within the Australian prawn farming industry (Spann *et al.*, 1997). The principle species affected is *Penaeus monodon*, the most common cultured species in Australia. However, experimental infection has also been reported in *Penaeus esculentus*, *Penaeus merguensis* and *Penaeus japonicus* within Australia (Spann *et al.*, 2000). The reported prevalence of chronic GAV infection in *P. monodon* approaches 100% of wild caught broodstock currently used to supply the Australian prawn farming industry (Cowley *et al.*, 2000). This has a flow-on effect with approximately 100% of postlarvae and juveniles from hatcheries and ponds testing positive for GAV (Walker *et al.*, 2001). The reverse transcription (RT)-nested polymerase chain reaction (PCR) developed for GAV is both specific and sensitive for the detection of GAV with as little as 10 fg of cDNA being able to be amplified and detected (Cowley *et al.*, 2000). However, the cost of this detection method is too high for many prawn farmers wanting to test large numbers of samples.

The aim of this chapter was to develop alternative, cheaper detection methods for GAV and by inference, other agglutinating viruses within prawns, i.e. YHV, to provide a quantitative assessment of viral load. This study investigates haemagglutination (HA) using chicken erythrocytes for GAV detection. Nadala *et al.* (1997) have previously used HA as a qualitative and quantitative detection method for YHV. YHV and GAV are indistinguishable with respect to morphological characteristics (Cowley *et al.* 1999) and since HA is caused by a reaction between the receptor on the erythrocyte and an external protein attached to the envelop of the virus (Figure 6.1), GAV should cause similar HA of chicken erythrocytes. This method is not as sensitive or specific as the RT- nested PCR. However the advantage

of this test is a qualitative and quantitative result with speed and ease of operation at a cost of AU\$ 0.03 per sample, enabling much larger sampling sizes. It also requires less complicated technology and training.

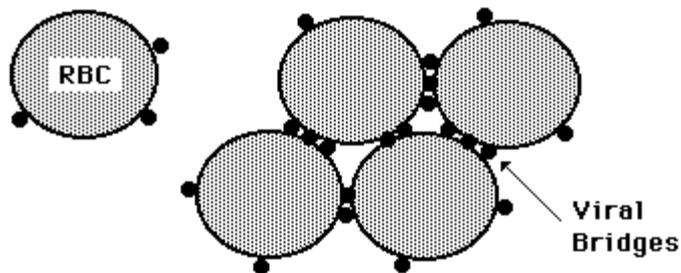


Figure 6.1. Reaction between the agglutinating virus and a receptor on the erythrocyte. Note: The virus can attach to more than one erythrocyte causing viral bridging indicating a positive result when testing for haemagglutination. RBC = red blood cell

6.2 Materials and Methods

6.2.1 Viral acquisition

Tissue from 100 black tiger prawns (12 – 17 g *P. monodon*), presumptively containing GAV, were obtained from a farm in northern Queensland, Australia. Either individual prawn organs or entire prawns were stored at – 20°C prior to HA.

6.2.2 Determination of optimal tissue/organ to obtain virus

The haemolymph, gill tissue, lymphoid organ, heart, hepatopancreas, subcutaneous tissue, eye stalk without the eye, pleopods, uropods and central nerve cord were removed from 100 individual prawns. The samples were then individually homogenised in 200 µl of phosphate buffered saline (0.1 M phosphate, 0.138 M NaCl, 2.07 mM KCl, pH 7.2) (PBS) and centrifuged at 16 000 × *g* for 20 min using an Eppendorf 5415D centrifuge with a fixed angle rotor. The supernatant was then removed for HA. The tissue/organ that was both easy to obtain and resulted in a relatively high HA titre was used for subsequent experiments.

6.2.3 Obtaining and storing chicken erythrocytes

Chicken blood was removed from the wing vein of an adult chicken using a 25-gauge needle. The blood was then stored in Alsever's solution (71.80 mM NaCl, 113.70 mM glucose, 27.70 mM tri-sodium citrate, 2.60 mM citric acid, triple distilled water) at 4°C for a minimum of 1 day and a maximum of 14 days.

6.2.4 Haemagglutination

Serial dilutions of prawn supernatant in PBS were mixed with equal volumes (50 µl) of 0.5% (v/v) chicken erythrocytes and then incubated for 2 h at room temperature. A reciprocal of the highest dilution at which the maximum agglutination occurred was recorded as HA titre.

6.2.5 Controls

In each experiment, both a negative and positive control for HA were used. The negative control was gill tissue from red claw crayfish (*Cherax quadricarinatus*) and the positive control was purified vaccine strain Newcastle disease virus (NDV). NDV is a *Paramyxovirus* which is known to readily cause HA of chicken erythrocytes.

6.2.6 Determination of the effect of freezing samples

To ensure that freezing and thawing of samples did not affect the HA protein on the virus, an experiment was conducted on gills from 120 *P. monodon*. Twenty of the gill samples were subjected to HA prior to freezing at -20°C. The remaining samples were frozen and periodically removed, thawed and refrozen. Each time the samples were thawed, 20 samples were removed and tested for HA until all samples had been used. This procedure gave a freeze/thaw cycle of 5 times.

6.2.7 Determination of natural agglutinins in *Penaeus esculentus*, *Penaeus merguensis* and *Penaeus longistylis*

The procedure previously described to determine optimal tissue/organ for HA (section 6.2.2) was utilised for these species, however, only gill tissue was utilised to determine HA titre from 20 prawns of each species.

These three penaeid species had no reports of natural infection of GAV and were tested to determine whether the negative control was a true representation of a negative result and that HA was due to GAV and not due to natural agglutinins present in penaeid prawns.

6.2.8 RNA isolation to determine prevalence of GAV

Total RNA was extracted following a modified method of Cowley *et al.* (2000). Briefly, a single gill filament was removed from 20 prawns for each species. These were then homogenised in 1000 µl of TRIzol (Life Technologies) and total RNA was isolated following the manufacturer's instructions.

6.2.9 Reverse transcription for GAV

The method to reverse transcribe the RNA to cDNA was adapted from Cowley *et al.* (2000) (Chapter 4.2).

6.2.10 PCR primers towards GAV

The primers used in this study for the detection of GAV were developed by Cowley *et al.* (2000). Briefly, the outer nest primers were GAV-5 (5'-AAT TTT GCC ATC CTC GTC AC-3') and GAV-6 (5'-TGG ATG TTG TGT GTT CTC AAC-3') amplified a 618 base pair (bp) region of the genome. The inner nested primers were GAV-1 (5'-ATC CAT ACT ACT CTA AAC TTC C-3') and GAV-2 (5'-GAA TTT CTC GAA CAA CAG ACG-3') and amplified a 317 bp region of the genome.

6.2.11 Statistical analysis

Analysis between groups was performed by one-way ANOVA and comparisons of individual means were compared using least significant difference (LSD) multiple comparisons with Statistical Package of Social Sciences (SPSS) for Windows version 10.

6.2.11 Animal Ethics

Ethics for this project was granted through James Cook University ethics review committee. Ethics approval number was: A746.

6.3 Results

6.3.1 Prevalence of GAV in studied penaeids

The prevalence of GAV in the studied populations of *P. esculentus*, *P. merguensis*, *P. longistylis* and *C. quadricarinatus* was 0% (0/20) for each species while the prevalence in *P. monodon* was 95% (19/20) by RT-nPCR.

6.3.2 Determining optimal tissue/organ from haemagglutination

The HA titres from organs and tissues of *P. monodon* were as a group, significantly different from each other ($F=71.37$, $DF=9$, 171 , $P<0.001$) (Figure 6.2). The only organs/tissues that had no significant difference were the lymphoid organ compared to the uropod ($P=0.142$), the heart compared to the subcutaneous tissue ($P=0.294$), the subcutaneous tissue compared to the central nerve cord ($P=0.094$) and the pleopod compared to the central nerve cord ($P=0.599$) (Table 6.1).

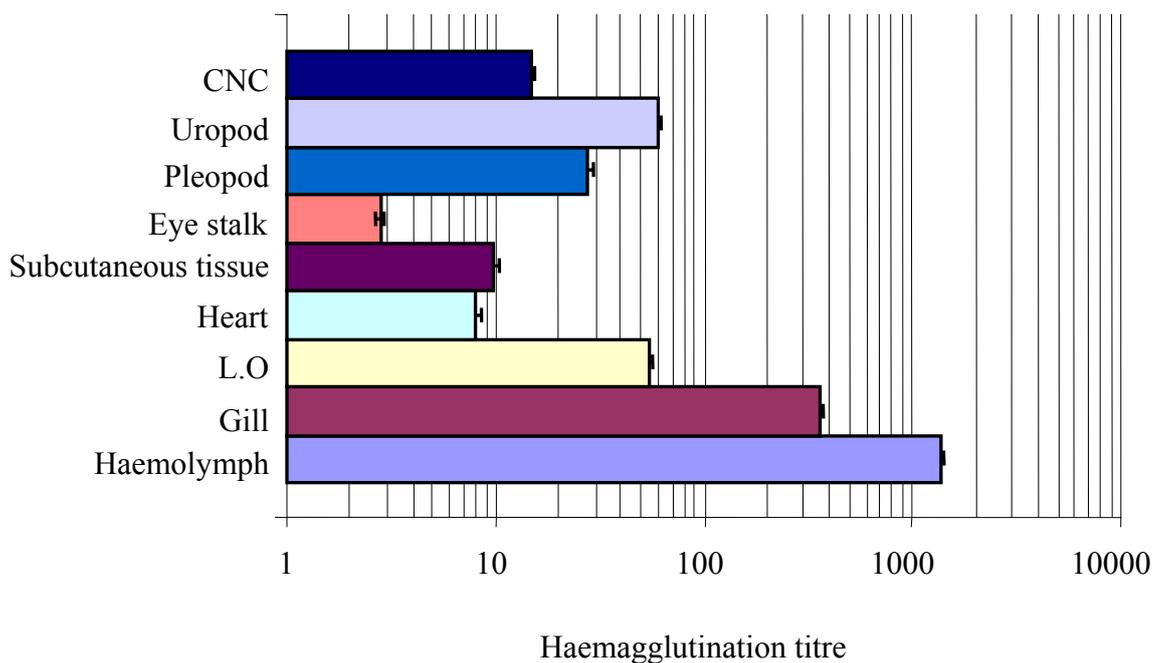


Figure 6.2. Haemagglutination titre of various organs/tissue from *Penaeus monodon*. Note: (CNC) central nerve cord. (L.O) lymphoid organ

Table 6.1. Significant differences (P value) of haemagglutination titre from individual organs/tissue compared to other individual organs/tissue from *Penaeus monodon*. Note: (L.O) lymphoid organ, (Tissue) subcutaneous tissue, (CNC) central nerve cord.

	Hemolymph	Gill	L.O	Heart	Tissue	Eye stalk	Pleopod	Uropod	Mean titre (log)
Hemolymph									9.9 +/- 0.42
Gill	0.000								7.9 +/- 0.35
L.O	0.000	0.000							4.9 +/- 0.39
Heart	0.000	0.000	0.000						2.2 +/- 0.29
Tissue	0.000	0.000	0.000	0.294					2.7 +/- 0.32
Eye stalk	0.000	0.000	0.000	0.007	0.000				0.9 +/- 0.26
Pleopod	0.000	0.000	0.022	0.001	0.028	0.000			3.8 +/- 0.43
Uropod	0.000	0.000	0.142	0.000	0.000	0.000	0.000		5.6 +/- 0.25
CNC	0.000	0.000	0.005	0.007	0.094	0.000	0.599	0.000	3.5 +/- 0.26

6.3.3 Determining haemagglutination titre from other penaeid prawns

There was a significant difference in HA titre between the different species of penaeid prawns ($F= 56.4$, $DF= 4, 88$, $P< 0.001$) (Figure 6.3). The only species that were not significantly different from each other were *P. esculentus* and *P. longistylis* ($P= 0.327$) (Table 6.2). It can also be seen from Figure 6.3 that the sample of *P. monodon* that was frozen had a lower haemagglutination titre than the fresh *P. monodon* sample.

HA results for the detection and indication of GAV load in four species of penaeid prawns, using gill tissue is shown in Figure 6.3.

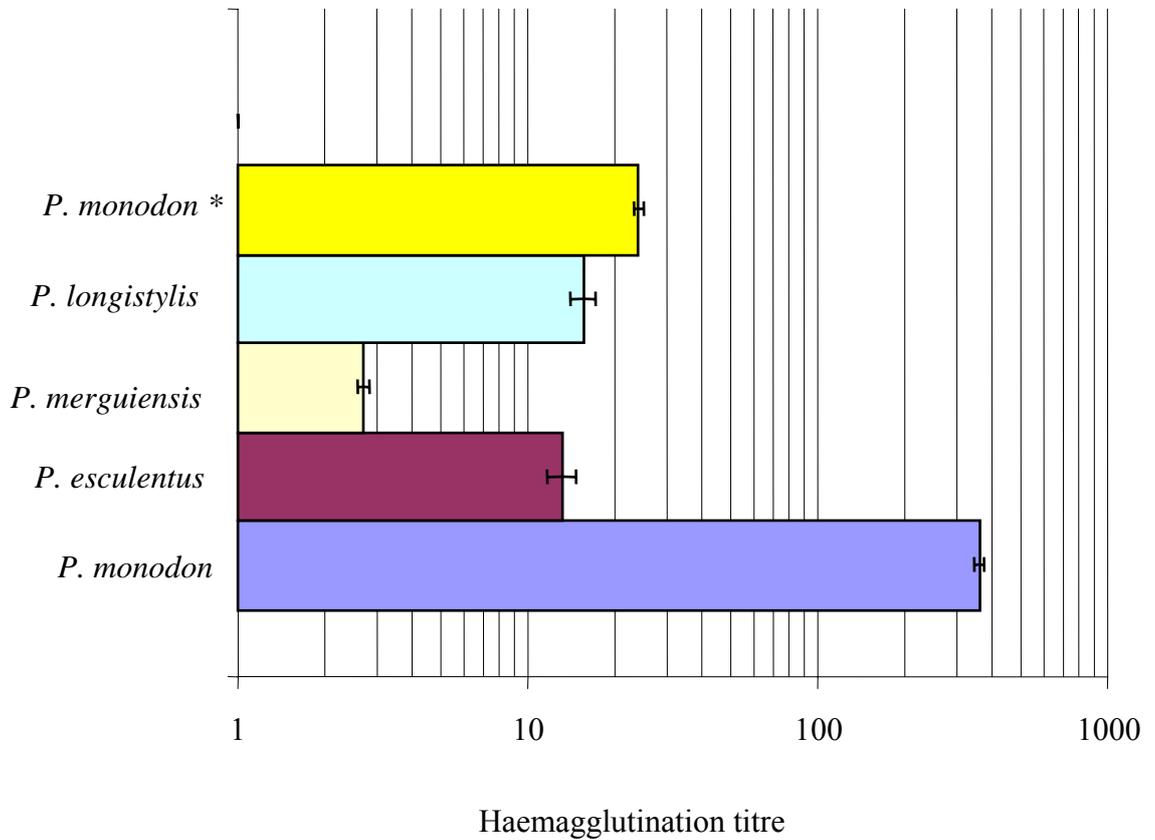


Figure 6.3 Haemagglutination titre of gill tissue from penaeid prawns. Note: (*) frozen *Penaeus monodon*

Table 6.2. Significant differences (P value) of haemagglutination titre using gill tissue between different species of penaeid prawns. Note: (*) frozen for unknown period

	<i>P. monodon</i>	<i>P. esculentus</i>	<i>P. merguensis</i>	<i>P. longistylis</i>	Mean titre (log ²)
<i>P. monodon</i>					5.5 +/-0.24
<i>P. esculentus</i>	0.000				1.4 +/-0.29
<i>P. merguensis</i>	0.000	0.041			0.7 +/- 0.17
<i>P. longistylis</i>	0.000	0.327	0.003		1.8 +/- 0.30
<i>P. monodon</i> *	0.000	0.000	0.000	0.004	2.9 +/- 0.21

6.3.3 Determining the effect of freezing samples on haemagglutination titre

There was no significant difference ($F = 0.241$, $DF = 1, 119$, $P = 0.625$) between the number of times the gill tissue was frozen and thawed with respect to HA titre (Figure 6.4). There was also a negligible association between the number of freeze/thaw cycles and HA with an R square value of 0.002 ($t = 0.491$, $P = 0.625$).

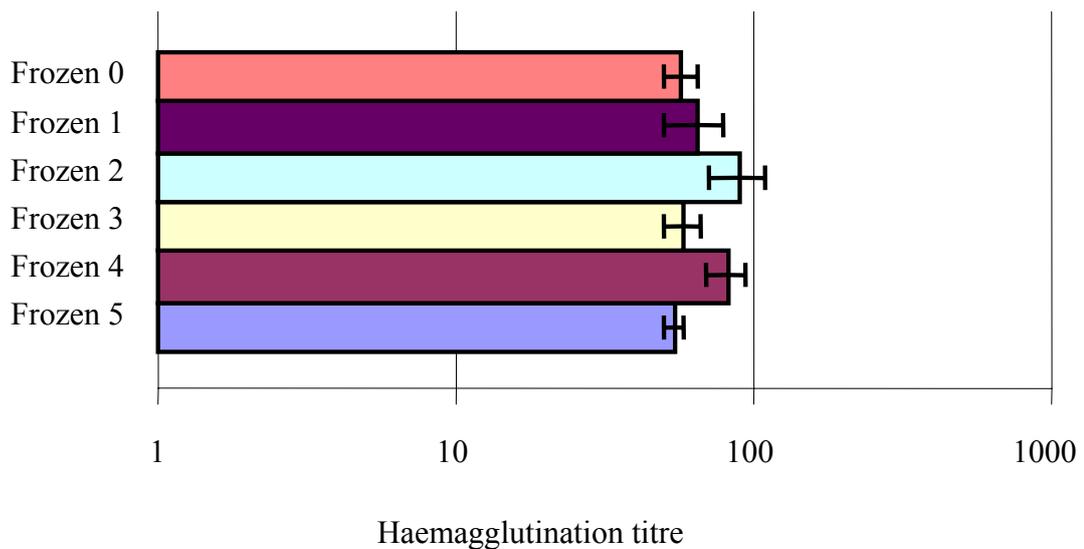


Figure 6.4 The effects of serial freezing of *P. monodon* gill tissue (n=20) on haemagglutination titre

6.4 Discussion

This chapter has described a quick, low cost, quantitative assay for the detection of GAV using HA of chicken erythrocytes. It has been reported that natural agglutinins are present in the haemolymph of *P. monodon* (Selvin, Huxley, and Lipton 2003) *P. longirostris* (Fragkiadakis and Stratakis, 1995), *Penaeus japonicus* (Muramoto, Matsuda, Nakada, and Kamiya 1995), *Penaeus californiensis* (Vargas-Albores, Guzman, and Ochoa 1993) and *Penaeus indicus* (Jayasree, 2001). In these papers they reported that natural agglutinins in the haemolymph cause agglutination towards various bacteria and to mammalian and fowl erythrocytes. Muramoto *et al.* (1995) is the only study that has tested haemolymph for natural agglutinins with chicken erythrocytes so this present study was primarily compared to their study.

In this current study, a HA end-point titre of 1:1370 was obtained compared to an endpoint titre of 1:2, which was reported by Muramoto *et al.* (1995). It is hypothesised that the difference in titre is due to the loading of GAV.

In addition, it was also reported that agglutination was influenced by the presence of calcium and magnesium ions (Muramoto *et al.*, 1995, Jayasree, 2001). The protocols that were used for this study were calcium and magnesium free.

Due to the possibility of high levels of natural agglutinins in the haemolymph, gill tissue was used for further studies since it is a completely different tissue, it should have low levels of natural agglutinins due to lower levels of circulating haemolymph and has high levels of GAV. From Figure 6.3 it can be seen that gill tissue from *P. esculentus*, *P. merguensis* and *P. longistylis* caused very low agglutination (1:2.7-15.6) compared to gill tissue from *P. monodon* (1:360.8). The low level HA in other penaeid prawns may be from contaminating haemocytes. While the high agglutination from *P. monodon* may be slightly increased by low level haemocyte contamination, it is thought that the majority of HA was from GAV. If natural agglutinins were present in the gill tissue of penaeid species then *P. esculentus*, *P. merguensis* and *P. longistylis* would all result in similar HA results as *P. monodon*. The only gross difference between *P. monodon* and the other three penaeid species is that *P. monodon* has a reported natural prevalence of GAV approaching 100% (Spann *et al.*, 2000) while there is no reported natural infection of GAV in the other three penaeid species. Figure 6.3 also demonstrates that different populations of *P. monodon* have different HA activity, possibly indicating a difference in viral load. This difference is not due to storage as no correlation was shown with respect to freezing and thawing of samples. In addition, closely related freshwater crayfish used as a control had no agglutinating ability (Results not shown). Therefore, it is hypothesised that the haemagglutinins in this report were largely from GAV, and not from innate crustacean immunity.

In summary, the logical evidence presented in this chapter suggests that the majority of agglutination caused from the gill tissue of *P. monodon* was from GAV. The other possible cause of the agglutination is that of the reported Mourilyan virus (MoV).

This virus has been classified as bunya-related virus. If the classification is correct then it also could be responsible for the agglutination of chicken erythrocytes, however, until there is published information on this virus i.e. genomic sequence, prevalence, host range etc, its affect on the HA test cannot be determined.

Nadala *et al.* (1997) previously used HA as a method for the detection of YHV. The two viruses, GAV and YHV are closely related, sharing 95.8% amino acid sequence and 85.1 % nucleotide identity (Cowley *et al.*, 1999) and are morphologically indistinguishable. With this information it is reasonable to assume that GAV would also agglutinate chicken erythrocytes. Nadala *et al.* (1997) reported that YHV agglutinated chicken erythrocytes, yielding a HA end-point titre of 1:256. In the study of Nadala *et al.*, (1997) they used purified YHV preparations from the gills, head soft tissue, and haemolymph of experimentally infected prawns. They reported that the haemolymph yielded the cleanest preparation of virus as determined by electron microscopy.

This study has shown that presumptive GAV from the gill tissue of *P. monodon* causes agglutination of chicken erythrocytes, that gill tissue from *P. esculentus*, *P. merguensis* and *P. longistylis* causes very low agglutination of chicken erythrocytes indicating very little natural agglutinins while crayfish gill causes no agglutination, and different populations of *P. monodon* gave different HA titres possibly indicating a difference in GAV load, and that freezing and thawing of samples five times had no effect on agglutination. It has been demonstrated that HA can be used as a low cost, low technology, quantitative test for the presence of GAV, and by inference for YHV when high sensitivity is not required. The test is easily applicable to on-farm testing of samples and it also allows samples to be frozen and tested at a later time since there was no correlation between freezing period or the number of freeze/thaw cycles with respect to HA.

In conclusion this assay is directed as an on-farm management tool in developing countries for the detection and estimation of YHV infection to better manage crops, since PCR is expensive and chicken erythrocytes are extremely easy to acquire. The test uses gills as the tissue sample, which excludes other possible agglutinating viruses. The other common systemic *P. monodon* viruses in developing countries are the infectious hypodermal and haematopoietic virus (IHHNV) and the white spot

syndrome virus (WSSV), both of which have no reported agglutinating properties towards chicken erythrocytes.

CHAPTER 7

SENSITIVITY AND SPECIFICITY OF CURRENT DIAGNOSTIC TESTS FOR GILL-ASSOCIATED VIRUS

7.1 Introduction

Gill-associated virus (GAV) was first reported in 1995 by Spann, Vickers, and Lester as its senior synonym, lymphoid organ virus (LOV) (Cowley *et al.*, 2000). The reported prevalence of chronic GAV infection in *Penaeus monodon* approaches 100% of wild caught broodstock (Cowley *et al.*, 2000) and approximately 100% of postlarvae and juveniles from hatcheries and ponds tested positive for GAV (Walker *et al.*, 2001). The reverse transcription nested polymerase chain reaction ((RT-nPCR) developed for GAV is both specific and sensitive for the detection of GAV (Cowley *et al.*, 2000). However, the cost of this detection method is too high for many prawn farmers wanting to test large numbers of samples.

Two low cost detection methods for GAV have recently been reported. Firstly, an ELISA using polyclonal (PAbs) and monoclonal (MAbs) antibodies for the detection of GAV has been produced (Munro and Owens submitted) (Chapter 5). Secondly, haemagglutination (HA) using chicken erythrocytes as a low cost detection method for GAV was reported by Munro and Owens (2005) (Chapter 6). Both of these methods provide a low cost, quantitative assessment of GAV load.

The aim of this chapter was to compare the three detection methods of GAV, namely RT-nPCR, ELISA and HA, with respect to sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy and to determine the best use of the tests by farm management.

7.2 Materials and Methods

7.2.1 Sample preparation of gill tissue for comparison between tests

One hundred and twenty *P. monodon* were collected from a prawn farm in northern Queensland for both comparison between the RT-nPCR and ELISA and the ELISA and HA. All the gill tissue from each prawn was removed and placed into labelled sterile microcentrifuge tubes and stored at – 80°C. For each prawn, one gill filament each was removed for both RT-nPCR and ELISA, while five gill filaments were removed for HA.

7.2.2 RNA isolation

Total RNA was extracted following a modified method of Cowley *et al.* (2000). Briefly, a single gill filament was removed from the prawn and homogenised in 1000 µl of TRIzol (Life Technologies) and total RNA was isolated following the manufacturer's instructions.

7.2.3 Reverse transcription (RT) – nested PCR

The method to reverse transcribe the RNA to cDNA and the subsequent cDNA amplification was adapted from Cowley *et al.* (2000).

7.2.4 PCR primers

The primers used in this study for the detection of GAV were developed by Cowley *et al.* (2000). Briefly, the outer nest primers; GAV-5 (5'-AAT TTT GCC ATC CTC GTC AC-3') and GAV-6 (5'-TGG ATG TTG TGT GTT CTC AAC-3') amplified a 618 base pair (bp) region of the genome. The inner nested 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') amplified a 317 bp region of the genome.

7.2.5 Semi-quantitative grading with RT-nPCR

The method used to determine the semi-quantitative viral loading of GAV was from Chapter 4.2. Briefly, the relative level of infection was derived from the intensities of

the visualised outer and inner nested PCR products (10 µl) on a 2% DNA-grade agarose-TAE gel (Progen Industries Ltd) containing 0.5 µl/ml ethidium bromide. The more intense the band from the agarose, the more DNA is present which is semi-proportional to the relative amount of viral RNA present in the total RNA. Thus, a larger viral load in the gills implies a higher percentage of viral RNA relative to the total RNA and thus an increased amplification of this viral RNA, which leads to a brighter band visualised on the agarose gel. The grading was as follows:

- 4 = strong outer nest (high viral load),
- 3 = weak outer nest (high/medium viral load),
- 2 = strong inner nest (medium/low viral load),
- 1 = weak inner nest (low viral load),
- 0 = no GAV detected (below detection limits).

7.2.6 Screening for GAV using a capture ELISA

Unless otherwise stated all quantities added to the ELISA plate wells were 50 µl in volume.

Chicken PABs against GAV were diluted at 1 in 80 in ELISA diluent (TropBio, Townsville, Australia) and were added to each well of a 96 well U bottom plate (Starstedt, Australia). This was then incubated overnight and washed once with TEN buffer (TropBio, Townsville, Australia). The plates were then blocked using 150 µl post-coating buffer (TropBio, Townsville, Australia) for 2 hours. A single gill filament from the posterior end of the prawn gills was homogenised and diluted in 200 µl of carbonate buffer (0.037 M NaHCO₃, 0.013 M Na₂CO₃) and centrifuged at 12 000 x g for 15 minutes. The supernatant was added to the ELISA plate wells and further incubated for 1 hour. The MAb (derived from mice) in cell culture fluid were added to the plates and further incubated for 1 hour. Goat anti-mouse IgG (H+L) – HRP conjugate (TropBio, Townsville, Australia) diluted to 1/120 in ELISA diluent was added and incubated as before for 1 hour. ABTS (2,2'-azino-di-3-ethylbenzthiazoline-6-sulphonic acid) was added and incubated for a further hour prior to determining the colour formation with the ELISA reader (Labsystems, Multiscan EX) using dual wave length of 414 and 492 nm.

7.2.7 Haemagglutination

The method used for HA was adapted from Munro and Owens (2005) (Chapter 6). Briefly, five gill filaments per prawn were homogenised in 200 µl of phosphate buffered saline (0.1 M phosphate, 0.138 M NaCl, 2.07 mM KCl, pH 7.2) (PBS) and centrifuged at $16\,000 \times g$ for 20 min (Eppendorf 5415D centrifuge with a fixed angle rotor). The supernatant was then removed for HA. Serial dilutions of prawn supernatant in PBS were mixed with equal volumes (50 µl) of 0.5 % (v/v) chicken erythrocytes in a 96 well U bottom plate and incubated for 2 h at room temperature. A reciprocal of the highest dilution at which the maximum agglutination occurred was recorded as the HA titre.

7.2.8 Obtaining and storing chicken erythrocytes

Chicken blood was removed from the wing vein of an adult chicken using a 25-gauge needle. The blood was then stored in Alsever's solution (71.80 mM NaCl, 113.70 mM glucose, 27.70 mM tri-sodium citrate, 2.60 mM citric acid, triple distilled water) at 4 °C for a minimum of 1 day and a maximum of 14 days.

7.2.9 Controls

RT-nPCR: A previously developed synthetic positive control (Munro *et al.*, 2005) (chapter 3) was used in all RT-nPCR reactions to ensure the reaction performed correctly. This synthetic control also eliminated false positives due to plasmid contamination. As a negative control, gill filaments from red claw crayfish (*Cherax quadricarinatus*) were used (all *C. quadricarinatus* in this experiment previously tested negative for GAV).

ELISA: Gill filaments from prawns that tested positive for GAV by RT-nPCR were used as positive controls, while the negative control was *C. quadricarinatus* gill (RT-nPCR negative for GAV).

HA: In each experiment, both a negative and positive control for HA were used. The negative control was *C. quadricarinatus* gill (RT-nPCR negative for GAV) and the positive control was purified Newcastle disease virus (NDV). NDV is a *Paramyxovirus* which is known to readily cause HA of chicken erythrocytes.

7.2.10 Comparison clarification

Sensitivity: The ability to detect those who truly have a disease.

Specificity: The ability to say a disease is absent when it is truly absent.

Positive predictive value (PPV): The ability of a test to predict the presence of disease.

Negative predictive value (NPV): The ability of a test to predict the absence of disease.

Accuracy: The proportions of all tests, both positive and negative that are correct.

7.2.11 Optical density cut off

The optical density cut off is the point below which a negative result is recorded.

7.3 Results

7.3.1 Comparison of the ELISA with RT-nPCR

Within the first experiment of comparing the ELISA with the RT-nPCR it can be seen that the RT-nPCR detected 83.3 % (100 / 120) of the prawns as being positive for GAV (Figure 7.1). As the optical density cut off for the ELISA was reduced, the percentage of prawns recorded as positive for GAV increased (Figure 7.2) while the overall optical density readings can be seen in Figure 7.3. The results obtained from using the different optical density as a positive cut off had a direct influence on the sensitivity, specificity, PPV, NPV, and the accuracy (Figure 7.4) of the ELISA. As it can be seen, the ELISA optical density cut off range of 0.5 to 0.6 resulted in the greatest sensitivity (98%) of the test while it produced the lowest accuracy (85.8 % and 86.7 % respectively). The optical density cut off of 0.75 resulted in the highest accuracy (91.7 %) and NPV (81.3 %) while it had the second highest sensitivity (97 %) and PPV (93.3 %). The optical density cut off of 0.9 had the highest specificity (80 %).

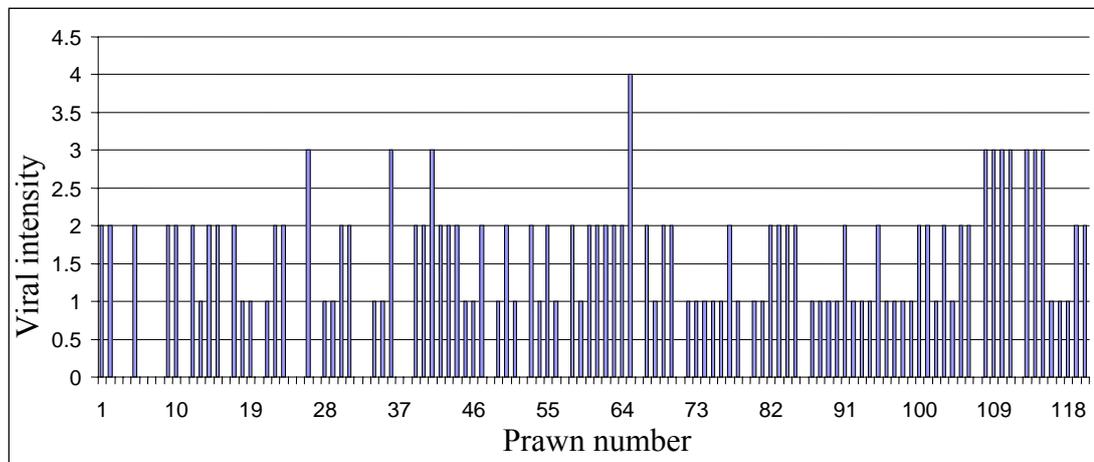


Figure 7.1. Reverse transcription nested polymerase chain reaction (gold standard) results from 120 *P. monodon* showing the prevalence and semi-quantitative viral load of GAV for comparison with the ELISA results (Figure 7.3)

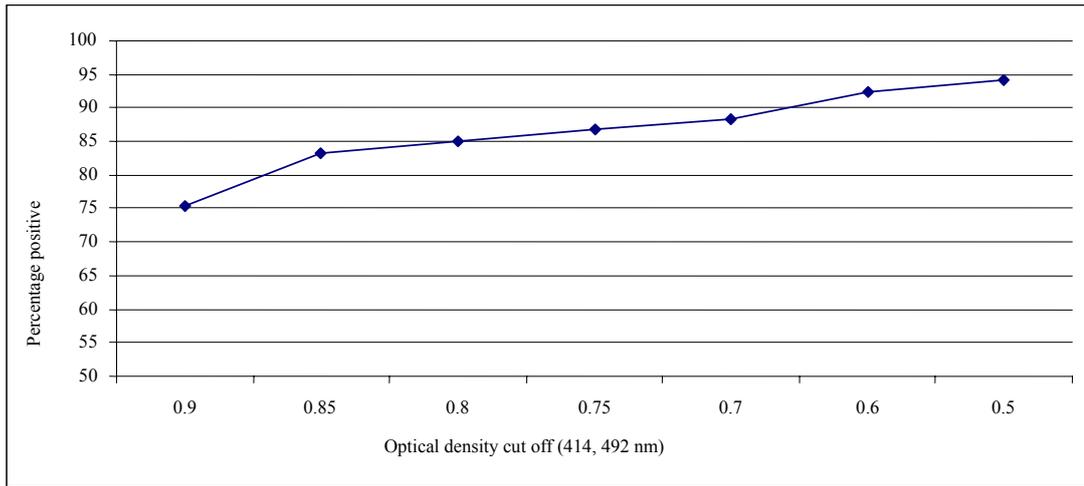


Figure 7.2. ELISA readings from 120 *P. monodon* showing the percentage of prawns recorded as positive at different optical density readings

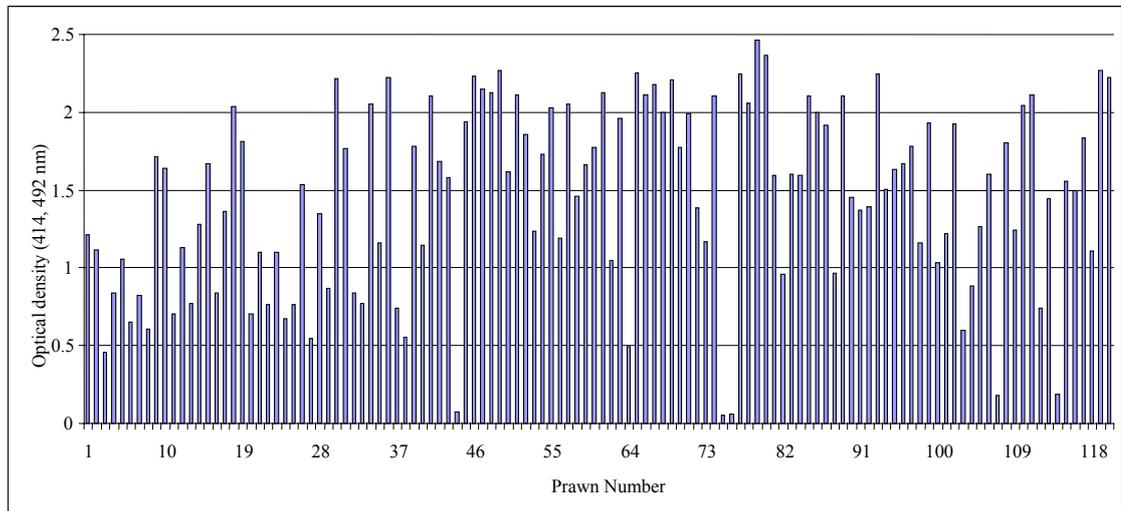


Figure 7.3. Prevalence and comparative level of infection of GAV when using the ELISA for GAV (n = 120) for comparison with the reverse transcription nested polymerase chain reaction results (Figure 7.1)

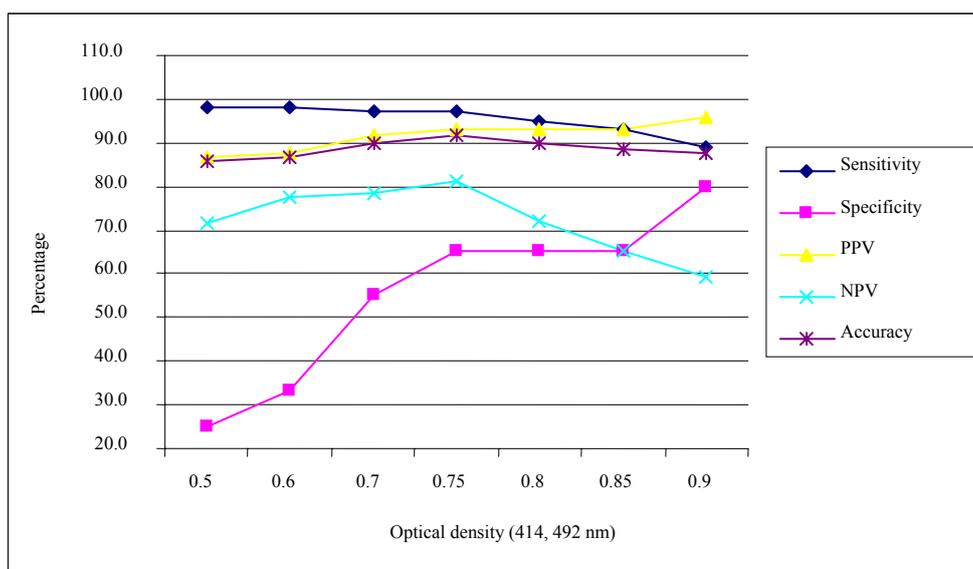


Figure 7.4. The sensitivity, specificity, positive predictive value, negative predictive value and the accuracy of the ELISA for GAV using different cut off values as a negative result when compared to the reverse transcription nested polymerase chain reaction

7.3.2 Comparison of haemagglutination with RT-nPCR

The comparison of the ELISA and the HA indicated that the ELISA resulted in 75% (90/120) of the prawns as being recorded as positive for GAV when using an optical density of 0.4 as a minimum reading (Figure 7.5). As the titre cut off for the HA increased, the percentage of prawns recorded positive for GAV decreased (Figure 7.6) while the overall titre readings can be seen in Figure 7.7. Using different HA titres had a direct effect on the results for, sensitivity, specificity, PPV, NPV, and accuracy (Figure 7.8) of HA.

The HA titre of 8 produced the highest sensitivity, specificity and NPV (94 %, 100 % and 100 % respectively), while the HA titre of 16 gave the highest accuracy (73 %) and the second highest specificity (75 %) and the HA titre of 64 gave the highest PPV (81 %).

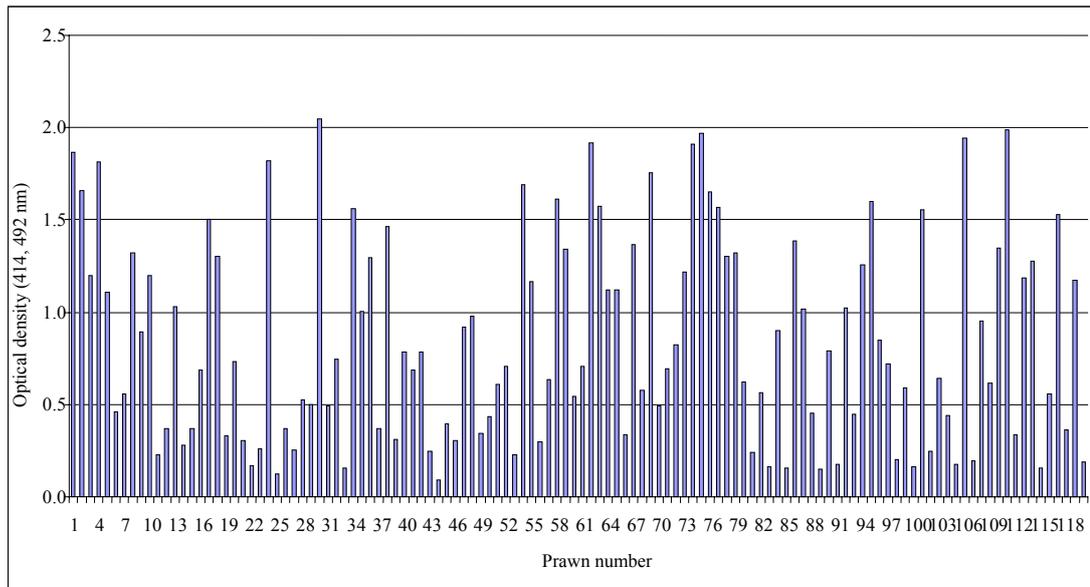


Figure 7.5. Prevalence and comparative level of infection of GAV when using the ELISA for GAV (n = 120) in comparison with the haemagglutination results (Figure 7.7)

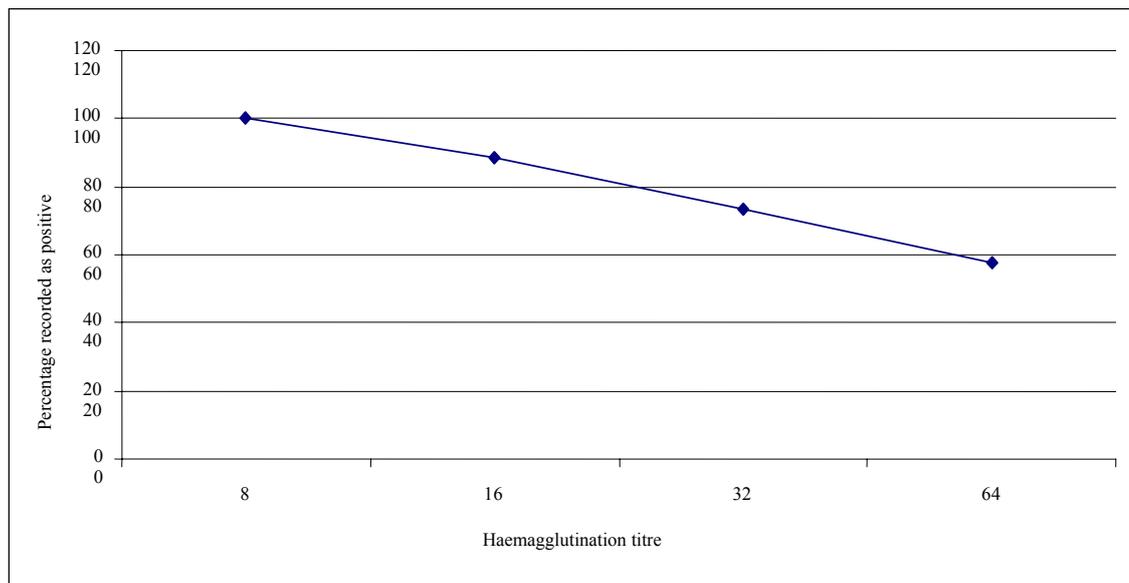


Figure 7.6. Showing the affect of increasing the haemagglutination titre cut off on the percentage of prawns that were recorded as positive for gill-associated virus

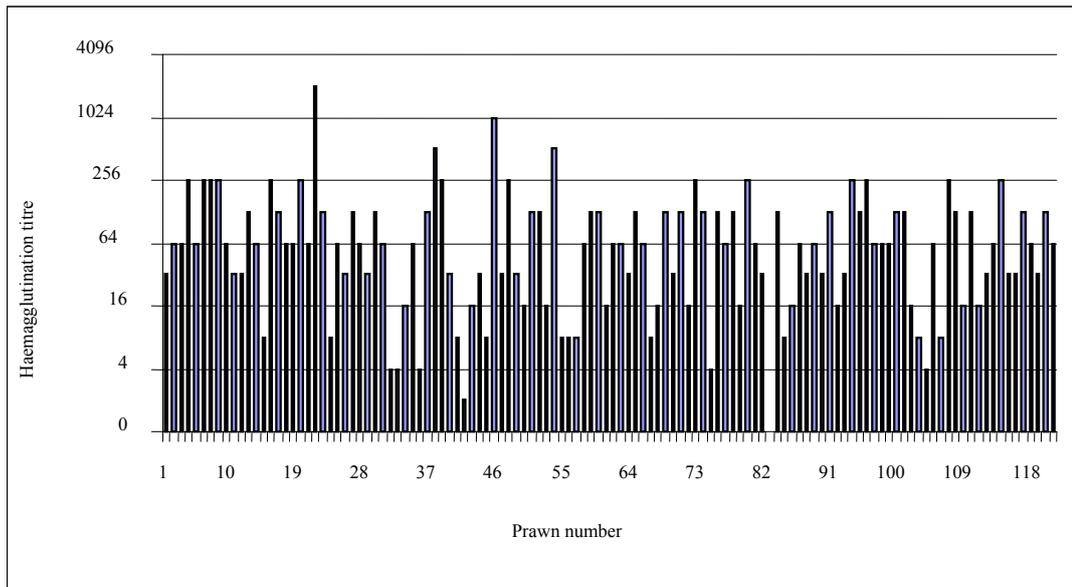


Figure 7.7. Prevalence and comparative level of infection of gill-associated virus when using the haemagglutination for the detection of GAV (n = 120) in comparison to the ELISA (Figure 7.5)

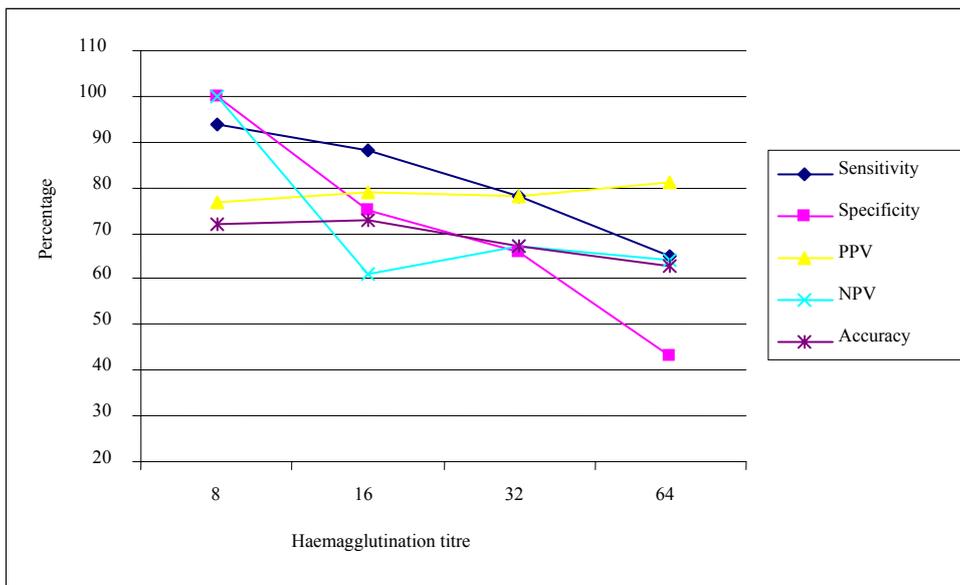


Figure 7.8. The sensitivity, specificity, positive predictive value, negative predictive value and the accuracy of haemagglutination for gill-associated virus using different titres to class negative results when compared to the ELISA. Note: Optical density used for ELISA was <0.4 = negative

7.4 Discussion

The comparison of the specificity and sensitivity of detection methods is common for determining the limitations of tests and the optimal test for different scenarios including cost effectiveness.

This chapter compared the sensitivity, specificity, PPV, NPV and the accuracy for the RT-nPCR with the ELISA and the ELISA with the HA assay for the detection of GAV. Currently, the routine method for the detection and screening of GAV is RT-nPCR. The high cost and skill required limit its' applicability to research institutes, government laboratories or large businesses. Based on costing analysis of 40 ponds with 20 samples/pond and two sampling periods, the RT-nPCR method costs \$27 200 for all consumable items while the ELISA costs \$6 400 for all consumable items and the HA costs \$48 plus the chicken erythrocytes. Due to the cost of the RT-nPCR, the test is often not feasible or cost effective for small business groups, resulting in blind management of GAV. The ELISA and HA both offer alternative low cost detection methods that when used under the correct management practices will produce equally valuable results as the RT-nPCR.

It can be seen that the cut off used to determine a negative sample has a direct effect on the sensitivity, PPV, NPV and accuracy. Thus, the required test and its cut off can be modified to suit the question that management is asking. For example, if broodstock were being screened for GAV and only GAV negative prawns could be utilised, then using HA with a cut off at a titre of 8 results in a specificity and NPV of 100 % compared to the ELISA. Likewise when using the ELISA, the sensitivity compared to the RT-nPCR varies from 89–98 % depending on the optical density cut off, with the higher optical density having the lowest sensitivity. However, if the management objective was primarily concerned with the samples that were truly positive, a high optical density cut off of 0.9 should be used, giving both the highest specificity and PPV. In comparison, if the management were concerned with samples with no GAV then the lowest optical density (0.5) cut off would be ideal. As can be seen by the low specificity, many samples would be false positives but this would ensure the chances of stocking without GAV would be maximised. If management wanted to know the general trend of disease on the farm, an optical density of 0.75 as a cut off appears to be the ideal medium with respect to the comparison factors,

particularly accuracy. Similarly, for HA the best all round performance occurs at a cut off of 16.

As this paper demonstrates, the type of test that is used does not necessarily depend on sensitivity or accuracy alone. Primarily, the test used has to be viable with respect to cost and sample size. Similarly, how the test is utilised or employed will depend on the question the management is asking.

CHAPTER 8

General Discussion

The production of aquatic animals from aquaculture has nearly tripled in the last ten years within developing countries of Southeast Asia (FAO Fishstat 2005). A considerable proportion of this aquaculture comes from prawn farming, which is practiced widely in Indonesia, Singapore, Malaysia, Vietnam and Thailand, and to a much lesser extent in Cambodia and Myanmar. Prawns from natural catches and aquaculture combined – are now the most important aquatic commodity in global markets, with prawn imports valued at more than US\$12 billion, or nearly 20 % of all international trade in aquatic animals (FAO Fishstat 2003). Diseases with viral aetiologies have been the most important cause of economic loss in the majority of countries (Fegan and Clifford 2001). With the three viruses, Taura syndrome virus (TSV), yellow head virus (YHV) and white spot virus syndrome (WSSV) being responsible for the most severe losses.

YHV and its close relative gill-associated virus (GAV) have been reported to be aetiological agents for causing mortalities in *Penaeus monodon*. Their pathogenicity has been based on the finding of histological lesions characteristic of the disease and/or the sole presence of either of these viruses. This assumption does not allow for the possibility that other viruses (such as WSSV) co-infecting the prawn cause similar lesions. Nor does it allow for the presence of multiple undetected viruses infecting the prawn which can individually have an effect on the mortality of the infected prawn.

Previous methods used to prevent and control GAV/YHV associated disease have generally involved the diagnosis and removal of infected prawns from the culture system. Molecular methods, such as reverse transcription nested polymerase chain reaction (RT-nPCR) and more recently, real time PCR have been designed for the detection of the two viruses. These molecular techniques are highly sensitive for the detection of the viruses; however, there are practical limitations to their utilisation. The requirement for specialised equipment, expensive molecular reagents and well-trained personnel pose as constraints for the widespread utilisation. Consequently,

these high costs in setting up and running the diagnostic tests result in the assay not being viable for the majority of prawn farmers or small support laboratories that screen for these viruses.

This present study has had four major outcomes. Firstly; a synthetic positive control has been developed for use with the RT-nPCR, enabling visual detection of plasmid contamination and thus, preventing false positives due to the plasmid. The implementation of this control when conducting RT-nPCR, has both enabled support laboratories to gain a higher level of confidence in their results and also facilitated accurate determination of the prevalence of GAV in three Australian farms, as part of a major study. Secondly, using RT-nPCR for detection, it was determined that both the prevalence and semi-quantitative load of GAV, have a strong association with production on Australian prawn farms.

While molecular techniques were successfully implemented in this study as part of a mass screening program for GAV, utilisation of this method would be limited due to its high expense. Therefore, two low cost detection methods, being an ELISA and haemagglutination (HA), were developed for the detection of GAV as the last two outcomes. These low cost methods will permit the implementation of disease screening strategies on small scale prawn farms in Australia and developing countries. These alternative detection methods have been compared with the RT-nPCR (as the gold standard) for their comparative sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and their accuracy of detection.

The developed synthetic PCR control worked correctly with both the outer and inner primers designed for the diagnostic amplification of 618 base pair (bp) and 317 bp products, respectively. This positive control produces a larger product than the diagnostic sample allowing easy visual discrimination. This technique is advantageous over the traditional method of cloning the diagnostic PCR product itself. Firstly, the positive control makes it visually easy to detect plasmid contamination and thus, prevent false positives from the plasmid. Secondly, the development of a positive control when the target sequence (viral, bacterial etc.) is at a very low prevalence can establish that the PCR conditions are correctly working

prior to the initial detection of the sequence. This will ensure the PCR is working correctly prior to diagnostic sampling, reducing false negatives. The traditional method of cloning the PCR product into a plasmid would rely on initial detection of the target sequence, which if at a low prevalence, could be time consuming and may not be possible in a novel field (where prevalence is unknown). Finally, the positive control enables development of a PCR and determination of its sensitivity, prior to the screening of diagnostic samples. The method used to produce this nested positive control demonstrates how to use large oligonucleotide primers in PCR without non-specific binding occurring.

From the three farms examined, GAV infection was found to be strongly associated with prawn production when RT-nPCR ($n = 1800$) was used as the detection method over the production period. Ponds with GAV-related outbreaks had a higher initial prevalence and a higher increase in prevalence. The ponds with no GAV outbreak – low production had the lowest initial prevalence but the largest increase in prevalence and the ponds with no GAV outbreak – high production had the lowest increase in prevalence.

The association of GAV with reduced production was also observed with the semi-quantitative viral loading of infected prawns. Ponds with GAV related outbreaks had the highest loading and percentage increase of loading at harvest. The ponds with no GAV outbreak – low production had moderate increase in GAV viral load and the ponds with no GAV outbreak – high production had the lowest increase in load. Prevalence and loading of GAV were associated with disease severity. Ponds with a higher initial prevalence and increase in GAV load suffered GAV-related outbreaks. Ponds with low initial prevalence of GAV but a high increase in prevalence and moderate increase in load over the production period, suffered low level mortality resulting in no outbreak being identified, yet low production. The ponds with moderate to low initial prevalence of GAV with a low increase in prevalence and load of GAV, incurred no GAV-related outbreak – high production. The findings of GAV being associated with disease are supported by Smith (2000) where he described degenerative and necrotic lesions associated with a GAV-like virus in the eye of *P. monodon* collected from an Australian prawn farm and concluded that this virus was a causative agent of disease. Callinan *et al.*, (2003) further added to the

study by Smith (2000). Callinan *et al.*, (2003) reported with the use of histopathology, immunohistochemistry and transmission electron microscopy (TEM) that GAV was the causative agent of disease outbreaks occurring in 22 of 25 ponds on a farm in eastern Australia. Callinan *et al.*, (2003) reported that the consistent demonstration by immunohistochemistry of a YHV-like virus in lesions in eyes and peripheral nerves of *P. monodon*, while no evidence of this virus in histological normal tissues in the same organs infers a causal relationship exists between GAV and disease outbreak. However it was not reported how many prawns were included in this trial.

The first low cost detection method developed in this study for the detection of GAV was an ELISA. This utilised polyclonal antibodies (PAbs) and monoclonal antibodies (MAbs) specific to GAV. Western blot analysis indicated that the PAbs reacted with 63, 110 and 170 kDa proteins, while the MAbs reacted to the 20 kDa protein of GAV. The MAbs were further investigated to ensure specificity by immunohistochemistry and elution of affinity purified GAV protein from the MAbs.

The second quantitative, low cost test was based on HA using chicken erythrocytes. This assay was developed to indicate the viral load of GAV, in *P. monodon*. The development of HA required testing of various types of prawn tissue to determine HA activity. It was found that the haemolymph and gill tissue produced the highest end-point titres of 1:1370 and 1:361, respectively. The sensitivity of the test was demonstrated by testing two different populations of *P. monodon*, which resulted in a highly significant difference ($P < 0.001$) in HA activity, indicating a difference in viral load. By testing three other penaeid prawn species ($n = 20$), *Penaeus esculentus*, *Penaeus merguensis* and *Penaeus longistylis*, and the crayfish *Cherax quadricarinatus*, it was demonstrated that natural agglutinins were not causing the high agglutination in the population of *P. monodon* being tested and thus producing the high values obtained. It was also demonstrated that there was no effect of freezing and thawing of samples on HA activity. The speed and low cost of this test makes it a very useful tool, particularly in developing countries, for on-farm testing of penaeid prawns to indicate YHV and GAV viral loads. This information can then contribute to management practices with respect to the appropriate timing for the harvesting of ponds.

The sensitivity, specificity, PPV, NPV and accuracy between RT-nPCR and an ELISA for the detection of GAV were determined. Subsequently, the same comparisons were applied between the ELISA and HA for the detection of GAV. The optical density or dilution cut off point had a direct influence on the test parameters. The cut off optical density of 0.5-0.6 with the ELISA produced a sensitivity of 98%. However, these optical densities produced the lowest accuracy. Utilising an optical density cut off of 0.75, resulted in the highest accuracy and NPV while it had the second highest sensitivity and PPV. However, the optical density cut off of 0.9 had the highest specificity.

With regards to HA, the dilution cut off at 8 resulted in the highest sensitivity, specificity and NPV, while the HA titre of 16 gave the highest accuracy and the second highest specificity, and the HA titre of 64 gave the highest PPV. Using the RT-nPCR as the gold standard, the ELISA had an accuracy of 91.7% when using an optical density of greater than 0.75 for a positive result, while the HA had an accuracy of 66.9 % when using an HA titre of greater than 16 for a positive result.

Currently, the detection method using RT-nPCR is the most specific and sensitive detection method available to small scale prawn farmers from Australia or developing countries. However, the best detection method is not necessarily the most sensitive or specific, it is the combination of affordability and the desired knowledge the farmer requires. RT-nPCR is the best detection tool for scientists with a large budget. However if the test is not cost effective, it does not matter how sensitive or specific it is, it is not an option as a detection tool for small scale farmers.

When using the RT-nPCR, ELISA, and HA based on a farm containing 40 ponds, with 20 sample prawns per sample and two sampling periods per crop, the farmer would have to have an increase in production equalling AUS\$127 200 for the use RT-nPCR to be cost effective, while an increase in production equalling AUS\$64 400 would be required when using the ELISA and an increase in production equalling AUS\$50 048 is required for the HA to be cost viable on Australian prawn farms (Table 8.1). This is based on laboratory set-up costs for each test and AUS\$50 000 for a full time technician to run the tests. The cost of the technician

would be reduced in developing countries and would vary on the level of skill required, i.e. RT-nPCR would require a high level of skill resulting in high labour costs, while the ELISA would require a moderate level of skill resulting in lower labour costs and the HA requires minimal skill levels resulting in very low labour costs. Therefore, in developing countries, which the HA is directed towards, the HA test would be extremely low cost compared to the RT-nPCR and ELISA. Likewise the ELISA would be much cheaper than the RT-nPCR.

Table 8.1. Cost analysis of reverse transcription nested polymerase chain reaction, enzyme linked immunosorbent assay and haemagglutination when used as detection tests for GAV based on 40 ponds, with 20 sample prawns/pond and two sample periods. Note:(*) = Technician cost can dramatically vary depending on the labour cost of individual countries.

	Sample cost (\$)	Set-up (\$)	Technician* (\$)	Total (\$)
RT-nPCR	27 200	50 000	50 000	127 200
ELISA	6 400	10 000	50 000	64 400
HA	0	48	50 000	50 048

These results indicate that the alternative detection methods for GAV (ELISA and HA) can be used to explore multiple questions about the disease status of the desired samples while still being cost effective and the “best” test to use is the test which gives the required sensitivity and specificity and more importantly being cost effective for the farmer.

In conclusion, this body of work has described the development of a plasmid PCR control that can be used in conjunction with diagnostic PCR to reduce false positives and negatives. By implementation of the synthetic positive control with the RT-nPCR it was demonstrated that an increase in prevalence and semi-quantitative load of GAV has a strong association with reduced production of *P. monodon* farmed within Australia. It cannot be concluded that GAV has a direct effect on production because no other viruses were tested for in this study. However, the conclusions drawn from this work are extremely strong. Without the availability of virus free *P. monodon* or a penaeid cell line with viable purified GAV the only stronger study that could be applied to this research is a systematic review, which would be extremely welcomed to test the findings of the current research. Other valuable

information that this study did not include was GAV prevalence and load from postlarvae in the hatchery or up to one month post-stocking. This information would be advantageous to determine the level of horizontal transition from the hatchery to stocking and to determine if it was a critical control point that could be used for monitoring. Also in this study, due to time and cost restraints only two sample periods were included. Increased sampling periods would help determine if there are peaks in GAV replication or if there is a linear increase in replication throughout the production period.

The development of HA and the ELISA provide farmers and support laboratories with greater options and increased viability when testing for the yellow head-like viruses. The comparison of the tests has demonstrated the level of confidence that the results are correct, and thus justifies their use as methods to screen for the yellow head-like viruses, GAV and YHV.

This research has a practical application for farmers by enabling them to have increased information to make management decision on the production of *P. mondon*. With the use of RT-nPCR, it is now possible to determine the likelihood of low production by sampling the prawns at one month poststocking, thus giving the farmer the opportunity to discard the crop and restock if they decided it would be beneficial. Alternatively, by sampling at one month poststocking and again closer to harvest, the likelihood of a GAV-related outbreak can be determined which can be used in the decision of when to harvest. The developed detection methods (HA and ELISA) could be used in hatchery situations. For example, if the manager wanted to used a subsample of broodstock which have the lowest GAV loading, then the HA assay or ELISA could be used to determine this and exclude broodstock with the highest loading at a fraction of the cost of using RT-nPCR. However, for the HA or ELISA to be used to predict a GAV outbreak, the two tests would need to be calibrated against the RT-nPCR so they could be used as a low cost monitoring tool on farms.

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APPENDIX 1 – Papers and Presentations

Papers

Munro J, Layton R, and Owens L. (2005) Development of a synthetic positive control which also detects plasmid contamination in diagnostic polymerase chain reaction. *Molecular Biology*. 39(6): 915-917

Munro J and Owens L. (2005) Haemagglutination as a low cost detection method for gill-associated virus and by inference, yellowhead virus in *Penaeus monodon* Fabricius, 1798. *Aquaculture Research* 36: 1369-1373

Munro J and Owens L. (2006) Sensitivity and specificity of current diagnostic tests for gill-associated virus. *Journal of Fish Diseases* 29: 649-655

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Munro J, Callinan R, and Owens L (Submitted) Gill associated virus is associated with decreased production of *Penaeus monodon* within Australian prawn farms. *Disease of Aquatic Organisms*

Munro J and Owens L. (Submitted) Yellow head-like viruses affecting the penaeid aquaculture industry – Review. *Aquaculture Research*

Presentations

J. Munro and L. Owens. “Alternative detection methods and epidemiology of the viruses involved in Mid-Crop Mortality Syndrome (MCMS)”. Australian Centre of International Agricultural Research, Darwin, December 2003.

J. Munro and L. Owens. “Development and delivery of practical disease control programs for small-scale shrimp farmers”. Australian Centre of International Agricultural Research, Bangkok, October 2004.

J. Munro and L. Owens. “Development and delivery of practical disease control programs for small-scale shrimp farmers”. 7th Diseases of Asian Aquaculture, Malaysia, December 2004.

J. Munro and L. Owens. “The effect of gill-associated virus on the production of *Penaeus monodon*”. FRDC Aquatic Animal Health Subprogram Scientific Conference, Cairns, July 2005.

J. Munro, L. Owens and R. Callinan. “The effect of gill-associated virus on the production of *Penaeus monodon* within Australian prawn farms”. 6th Symposium of Disease in Asian Aquaculture, Sri Lanka, October 2005.