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

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June 2006

for the degree of Doctor of Philosophy in the

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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 Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

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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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James Munro 06/03/2006

ACKNOWLEDGMENTS

Firstly, I would like to thank my supervisor Leigh Owens, not only for his support and enthusiasm for this project but also for giving me a lot of freedom in my PhD to investigate different aspects of the project.

I would like to thank Dick Callinan for helping to direct the project and obtaining funding from ACIAR. Without him, the project would never have been possible. I always enjoyed our meetings and found his overwhelming support for the project, inspiring.

I would like to thank Graham Burgess and Jan Smith for their help with questions I often had about antibodies and proteins.

The graduate students definitely need a big acknowledgement. Especially my fellow suffering room mate Kezza (a.k.a Crofty). Thanks to you, Court and Gin, I am sure my name will always be a legend in the Equatorial Hotel, Penang.

I would also like to give a big thank you to my sidekick and partner in crime, Ginny. Your support throughout my PhD, brainstorming and proof reading was amazing. Outside of uni, your companionship was as equally enjoyed.

I would like to thank my parents for their support, not only through my PhD but throughout my entire university education. Without their support I would never have been able to complete it.

Last but definitely not least, I would like to thank Dominion coffee shop. After a soured friendship with Hungry Jacks (according to the experts it was due to a gluten allergy, however I think we just grew apart), you were always there to fill a void. Without you I would have found it hard to gather the enthusiasm for this PhD.

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 merguiensis* 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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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-butylphethalate polysyrene 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
g	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 antbodies
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