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Aquatic Animal Health Subprogram:

Viral presence, prevalence and disease management in wild populations of the Australian Black Tiger prawn (*Penaeus monodon*)

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Abbreviations

AAHL	Australian Animal Health Laboratory (Geelong)
AFDL	AAHL Fish Disease Laboratory
APFA	Australian Prawn Farmers Association
bp	base pairs
CCEAD	Consultative Committee on Emergency Animal Diseases
cDNA	Complementary DNA
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DNA	Deoxyribonucleic acid
FRDC	Fisheries Research and Development Corporation
GAV	Gill-associated virus
GoC	Gulf of Carpentaria
HP	hepatopancreas
HPV	Hepatopancreatic parvovirus
IHHNV	Infectious hypodermal and haematopoietic necrosis virus
IMNV	Infectious myonecrosis virus
JBG	Joseph Bonaparte Gulf
MBV	Monodon baculovirus
MCMS	Mid-crop mortality syndrome
MoV	Mourilyan virus
nPCR	nested PCR
NQ	North Queensland
NT	Northern Territory
nt	nucleotides
OIE	World Organisation for Animal Health
PCR	Polymerase chain reaction
PL	Postlarvae
QBP	Queensland Bioscience Precinct
QDAF	Queensland Department of Agriculture and Fisheries
QLD	Queensland
rDNA	ribosomal DNA
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-PCR
RT-nPCR	RT-nested PCR
RT-qPCR	RT-quantitative PCR
SMV	Spawner-isolated mortality virus
TNA	Total nucleic acid
WSSV	White spot syndrome virus
YHV	Yellow head virus

Executive Summary

What the report is about

Reported here are the outcomes of a project to (i) test for enzootic and exotic viruses in wild stocks of Black tiger prawns (*Penaeus monodon*) inhabiting disparate regions in northern Australia and (ii) develop new PCR tests and appropriate test controls to improve upon the specificity of existing PCR tests for the exotic highly-pathogenic yellow head virus genotype 1 (YHV1). *P. monodon* were examined from locations in North Queensland (NQ), the Gulf of Carpentaria (GoC) and Joseph Bonaparte Gulf (JBG). Of the enzootic viruses targeted, there was a particular focus on Gill-associated virus (GAV) and a unique YHV genotype (YHV7) discovered recently in *P. monodon* originating from JBG. Testing for exotic viruses was conducted to provide additional evidence to support Australia's claims of pathogen freedom. Aspects of the project were undertaken within the Aquaculture Program, CSIRO Agriculture, Queensland Bioscience Precinct (QBP) and within the CSIRO Australian Animal Health Laboratory (AAHL) Fish Diseases Laboratory (AFDL).

Background

Two of the project objectives were undertaken to support the Australian Prawn Farmers Association, the Queensland Department of Agriculture, Fisheries and Forestry and commercial broodstock suppliers; to obtain data on the presence and prevalence of YHV genotypes (Objective 1) and other enzootic viruses in wild *P. monodon* (Objective 4). Regions targeted for sampling had been used or were being considered for use as a source of wild broodstock No systematic studies of what viruses could be inadvertently introduced via broodstock sourced from either NQ or more remote locations such as Joseph Bonaparte Gulf (JBG) or the Gulf of Carpentaria (GoC) have been undertaken. The need to determine the disease status of wild-caught broodstock was further highlighted after the discovery of YHV7 in *P. monodon* during a disease investigation in a hatchery in NQ, associated with broodstock translocated from the JBG.

The other two project objectives were undertaken to revise GAV/YHV molecular assays (Objective 2) and develop synthetic positive control material (Objective 3). In 2012, PCR testing of moribund *P. monodon* at a farm in Queensland (QLD) produced unexpected results with the OIE YHV protocol 2 test. When sent to AFDL for confirmatory testing the OIE YHV/GAV Protocol 2 reverse transcription (RT)-nested PCR (RT-nPCR) test produced anomalous false-positive results for YHV1 in the nested PCR (nPCR). Sequencing identified amplicons derived from GAV. Due to the identified lack of specificity of the test and concerns that this could lead to erroneous reporting of YHV1, it was identified as a high priority to refine or develop yellow head complex PCR tests.

Aims/objectives

The project had 4 objectives:

1. Determine what GAV/YHV genotypes exist and their relative prevalence in *P. monodon* populations in wild NT and QLD populations from which broodstock are captured for aquaculture purposes.
2. Revise PCR test designs as necessary to ensure their specificity, particularly in discriminating the highly virulent YHV-1 strain that emerged in Thailand in the early 1990s from GAV and the other

known YHV genotypic variants that appear to be far less pathogenic, and make these tests available for publication in the OIE Diagnostic Manual for yellow head disease.

3. Acquire and/or prepare appropriate control nucleic acids for the various YHV genotypes suitable for use with the YHV- or other genotype-specific tests so that their diagnostic performance can be validated at key testing laboratories such as CSIRO-AAHL, and for distribution to state and international laboratories with needs for equivalent diagnostic capabilities.
4. Determine the existence and prevalence of other endemic viruses [e.g. Mourilyan virus (MoV), Monodon baculovirus (MBV), Hepatopancreatic parvovirus (HPV), Spawner-isolated mortality virus (SMV) and Infectious hypodermal and hematopoietic necrosis virus (IHHNV)] in wild *P. monodon* populations in the NT and QLD. In addition, test samples for the exotic viruses WSSV and IMNV given some broodstock are sourced from waters with a higher than usual likelihood of incursion from these pathogens.

Methodology

Samples of *P. monodon* from populations inhabiting various locations in NQ, the GoC and JBG were sourced from either hatcheries, directly from a commercial broodstock collector or from fisheries survey trawlers. The goal was to collect appropriate tissues from at least 150 prawns from each region to allow viruses to be detected at 2% prevalence level, with 95% confidence assuming a perfect test. To accommodate the fact that unique genetic variants are known to exist for specific viruses (YHV/GAV, MBV, HPV, and IHHNV), available PCR tests were used where appropriate or PCR tests were designed at the CSIRO-QBP. Due to the recent discovery of YHV7, TaqMan and conventional PCR tests specific for this genotype were also designed and evaluated. Due to the deficiencies identified with the OIE YHV PCR tests, new YHV1-specific TaqMan and conventional PCR tests were also developed and evaluated.

Results/key findings

The exotic viruses (WSSV, IMNV, or YHV1) were not detected in any of the Australian-sourced *P. monodon* originating from any of the locations examined.

GAV was detected among *P. monodon* originating from all NQ study locations. For some prawns originating from Etty Bay (the most southerly NQ location examined) the PCR product sequence obtained was consistent with YHV6. This genotype is most closely related to GAV (= YHV2). Archived sequences derived from PCR products amplified from NQ *P. monodon* were re-examined and results indicated that YHV6 has existed in this region since at least the early 2000s (JA Cowley, unpublished). The GAV TaqMan real-time RT-quantitative PCR (RT-qPCR) detected GAV among *P. monodon* originating from JBG, confirmed by sequence analysis of PCR products. While GAV detections were made in some *P. monodon* from GoC using the GAV TaqMan real-time qPCR test, these detections were not confirmed by conventional PCR.

With regard to YHV7, detections were made using the YHV7 TaqMan real-time qPCR test in three prawns originating from NQ locations and in one prawn originating from a GoC location. These could not be confirmed by conventional PCR. The YHV7 TaqMan real-time qPCR test detected YHV7 in *P. monodon* originating from JBG with confirmation by conventional PCR and sequence analysis. YHV7 was also detected with confirmation by sequence analysis from broodstock and progeny from a hatchery in NQ.

Molecular assays to specifically detect YHV1 were designed and evaluated. Two assays, one in a format for high-throughput screening (YHV-1 TaqMan real-time RT-qPCR test) and one for confirmatory testing (YHV RT-nPCR test) showed appropriate analytical specificity and sensitivity. Limited availability of YHV1 known-positive material has delayed determination of diagnostic sensitivity and technology transfer of the assays to state laboratories. However, requests have been made to the international community for additional YHV1-positive samples. Samples generated through FRDC 2015-005 "Aquatic Animal Health Subprogram: Determining the susceptibility of Australian *Penaeus monodon* and *P. merguiensis* to newly identified enzootic (YHV7) and exotic (YHV8 and YHV10) Yellow head virus (YHV) genotypes" will be used for PCR test validation including tests for YHV1 which will be used as a positive control for infectivity trials. A number of published assays for detection of YHV1 and other YHV-complex genotypes (e.g. GAV) were also evaluated during the project. A lack of specificity was identified indicating the need for improvement of a number of these assays. With regard to YHV7, TaqMan real-time qPCR and conventional nPCR tests were designed and found to be specific for this genotype. Material for validation of the YHV7 assays will also be developed through FRDC 2015-005.

As validation of the new YHV1 and YHV7 TaqMan real-time qPCR and RT-nPCR tests has not been completed, positive control material was not developed and distributed as planned. As more extensive validation is undertaken, assays may need minor modifications which will negate the use of any controls developed. Assays can be transferred to state laboratories with tissue-derived positive controls now, if requested. Development of this material will be completed and distributed to state laboratories through FRDC 2014-002 "Aquatic Animal Health Subprogram: Development of stable positive control material and development of internal controls for molecular tests for detection of important endemic and exotic pathogens".

The MoV TaqMan real-time qPCR test detected MoV at low to high prevalence among prawns originating from various NQ locations. For SMV, only two of 151 NQ prawns and three of 143 JBG prawns tested positive. However, it is possible the SMV prevalence was an underestimated due to the unavailability of hepatopancreas (HP) tissue for most of the prawns tested. For IHHNV (excluding virus-like homologues in the host genome), PCR detections were made among prawns from each of the three regions studied. For MBV, only two of 151 NQ prawns and one of 37 GoC prawns tested PCR-positive. However, it cannot be discounted that the use of gill DNA resulted in the prevalence of MBV being underestimated. For HPV, PCR detections were made within the 3 study regions. However, the unavailability of HP tissue from prawns captured at some locations might have led to HPV prevalence being underestimated at these locations.

Implications for relevant stakeholders

The APFA supported the testing for exotic viruses and will be reassured as WSSV, IMNV and YHV1 were not detected in any of the *P. monodon* tested from any of the study locations. The 2012 discovery of YHV7 in broodstock originating from JBG was confirmed in 2013 samples from QLD hatcheries. However, as YHV7 was also detected and confirmed in QLD grow-out prawns, targeted surveillance of farms in QLD is required to determine the true prevalence and distribution of this genotype. Whether the detection of YHV7 in prawn farms in QLD is due to recent introductions with broodstock sourced from the Northern Territory or is due to YHV7 already being present in QLD prawns is unknown.

When validated, the revised YHV1 and YHV7 PCR tests and non-infectious synthetic artificial controls will be valuable for State Government diagnostic laboratories as well as commercial diagnostic laboratories undertaking certification testing for imported unprocessed prawns.

Recommendations

To assess what risks are posed by the YHV7 genotype, its pathogenicity to farmed prawns needs to be determined. This will be undertaken as part of FRDC 2015-005 “Aquatic Animal Health Subprogram: Determining the susceptibility of Australian *Penaeus monodon* and *P. merguiensis* to newly identified enzootic (YHV7) and exotic (YHV8 and YHV10) Yellow head virus (YHV) genotypes”. This project will also develop additional material required for validation of YHV1 and YHV7 assays and preparation of synthetic positive control material.

In order to determine the current prevalence of YHV7 in QLD farmed prawns and to clarify the translocation risk associated with broodstock from the Northern Territory, additional targeted surveillance should be undertaken of prawns in QLD.

Keywords

Black tiger prawns, *Penaeus monodon*, yellow head virus, gill-associated virus, enzootic viruses, exotic viruses, PCR tests and controls, northern Australia, aquaculture

Introduction

Yellow head virus genotype 1 (YHV1) is the causative agent of yellow head disease (YHD) and is the only yellow head complex genotype listed by the World Organisation of Animal Health (OIE). YHV1 emerged in the early 1990s as the cause of mass mortalities of *Penaeus monodon* farmed in Thailand (Chantanachookin *et al.* 1993). YHV1 continues to cause farm production losses in Thailand and has become established as a pathogen in farmed Pacific white prawns (*P. vannamei*) (Senapin *et al.* 2010). YHV1 has also been reported in other prawn farming regions such as Chinese Taipei, Indonesia, Malaysia, the Philippines, Sri Lanka and Vietnam (OIE, 2014). While YHV1 is exotic to Australia, a genotypic variant named gill-associated virus (GAV), synonymous with yellow head virus genotype 2 (YHV2), was discovered not long after the discovery of YHV1 (Spann *et al.* 1997, Cowley *et al.* 1999). GAV is enzootic at high prevalence in wild and farmed *P. monodon* in eastern Australia and can cause disease on farms (Cowley *et al.* 2000, Munro *et al.* 2011).

In addition to YHV1 and GAV, four other unique YHV genotypes were identified in healthy *P. monodon* collected across the Indo-Pacific region between 1997 and 2004 (Wijegoonawardane *et al.* 2008a). The new genotypes were described based on both the phylogeny of an ORF1b gene sequence and geographic location (e.g. YHV3 in Thailand and Vietnam; YHV4 in India; YHV5 in Malaysia, Thailand and the Philippines; and YHV6 in Mozambique).

The OIE Manual of Diagnostic Tests for Aquatic Animals currently recommends 3 PCR tests to detect and classify YHV genotypes (OIE, 2014). The OIE YHV Protocol 1 reverse transcriptase-polymerase chain reaction (RT-PCR) was designed to detect YHV1 (Wongeerasupaya *et al.* 1997). The OIE YHV/GAV Protocol 2 RT-nested PCR (RT-nPCR) is a more sensitive multiplexed test designed to detect and differentiate YHV1 and GAV (Cowley *et al.* 2004). The OIE YHV Protocol 3 RT-nPCR was designed using degenerate PCR primer sequences to amplify and classify, through sequence analysis, any of the six known YHV genotypes (Wijegoonawardane *et al.* 2008b).

Development of a FRDC project application emanated from discussions with key prawn industry representatives at the 2012 Australian Prawn and Barramundi Farmers Conference. This discussion focussed around the lack of recent knowledge about the infection status of Gill-associated virus (GAV) in wild *P. monodon* broodstock captured from various locations to supply hatcheries. Also discussed was broodstock captured from the east coast of NQ may pose a risk to their enterprises due to GAV infection. Together with evidence that broodstock pose a risk of transmitting infection to progeny (Cowley *et al.* 2002), and GAV-infected postlarvae (PL) being more at risk of disease during pond grow-out, many hatcheries/farms have been increasingly sourcing broodstock from the Joseph Bonaparte Gulf (JBG). As well as reducing disease problems, the use of JBG broodstock has also been adopted for their generally high fecundity and their progeny proving to be robust with fast growth during grow-out.

Combined with several facilities domesticating and selectively breeding high growth-performance *P. monodon*, the increased use of JBG broodstock has led to diminished requests for broodstock from specialist NQ broodstock suppliers. Concern was expressed that these factors had marginalised their commercial viability to a point whereby they could no longer guarantee fulfilling sporadic but often urgent requests for broodstock. Based on this discussion, it was suggested that the APFA would financially support research to (i) update data that are over a decade old on the prevalence of GAV

in NQ *P. monodon* and (ii) determine the prevalence of GAV in JBG *P. monodon* broodstock being imported for use in QLD hatcheries.

In late 2012, PCR testing undertaken by the Queensland Department of Agriculture and Fisheries (QDAF) identified the possible presence of an exotic YHV genotype in moribund *P. monodon* at a farm in QLD. Samples were submitted to the AAHL Fish Diseases Laboratory (AFDL) for confirmatory testing and were positive when tested using a modification of the OIE YHV/GAV Protocol 2 RT-nPCR test. An amplicon of the size expected for GAV was generated however, an amplicon was also produced with the test designed to be specific for YHV1. Sequencing identified both amplicons as GAV. Due to concerns regarding reporting of false-positive test results in investigations for YHV1, the Yellow Head Disease Chapter in the OIE Manual of Diagnostic Tests for Aquatic Animals was updated in 2013 with a recommendation that all PCR products generated using the OIE YHV/GAV Protocol 2 RT-nPCR be sequenced to confirm the virus genotype.

As part of the same disease investigation, QDAF tested *P. monodon* broodstock originating from Joseph Bonaparte Gulf (JBG) with mortalities that began to occur from day 1 post-receipt by the QLD hatchery. Again due to QDAF results raising suspicion of the presence of an exotic YHV genotype, samples were submitted to AFDL for confirmatory testing. Sequence analysis identified a YHV genotype dissimilar to any of the six genotypes (YHV1-6) described previously. Thus, this seventh distinct genotype was designated YHV7 (Mohr *et al.* 2015). The distribution of YHV7 in northern Australia and the involvement of this newly-detected YHV genotype in the disease episode that was being investigated are unknown.

In 2014 several imported unprocessed commodity prawn consignments from China tested PCR positive at a laboratory screening the prawns for YHV1 with the CSIRO YHV TaqMan RT-qPCR test before their release from quarantine. Positive tissues were submitted to AFDL for confirmatory testing. The samples tested negative for YHV with the OIE YHV/GAV Protocol 2 RT-nPCR test and the OIE YHV Protocol 3 RT-nPCR test but tested positive for YHV using the CSIRO YHV TaqMan RT-qPCR test. Due to the ambiguity of the PCR test data, the Australian Government Department of Agriculture (DoA) requested further investigation. Sequences of the PCR products amplified from the consignments were similar to one another but distinct from any of the recognised YHV1 to YHV7 genotypes. This newly-detected eighth YHV genotype was designated YHV8.

During the AFDL investigation of YHV8, GenBank sequence searches identified submissions (KF278563 and the shorter KF278564 and KF278565 sequences, Liu *et al.* 2014) of a YHV genotype detected in *Fenneropenaeus chinensis* and *Litopenaeus vannamei* in China. Sequence analysis of YHV8 showed it to be distinct from the Chinese sequence (KF278563) or any of the other described YHV genotypes. The KF278563 sequence submitted to GenBank by Chinese scientists was thus designated YHV genotype nine (YHV9) for convenience.

Following Australian Government DoA being notified of the detection of YHV8 (and the identification of YHV9) a protocol was instigated requiring tissues from all imported prawn consignments that tested positive for YHV to be provided to AFDL for confirmatory testing and genotyping. Samples from a further five prawn consignments from China were sent to AFDL for testing during 2014. Each batch tested negative for YHV1 using the OIE YHV/GAV Protocol 2 RT-nPCR, however, YHV8 was detected in four of the consignments. In the remaining consignment, sequences were significantly

different to those of YHV1 to YHV9. This newly-detected YHV genotype was thus designated YHV10. The species of prawns in which YHV8 and YHV10 were detected have yet to be confirmed.

Due to the detection of YHV7 and identification of specificity issues associated with the OIE YHV/GAV Protocol 2 RT-nPCR test during the disease investigation in the QLD farmed prawns, development of an FRDC Tactical Research Fund (TRF) project application was submitted and funded. Objectives included reassessment of the specificity of OIE PCR tests, particularly in relation to their potential to cross-detect currently circulating strains of GAV as well as other genotypic variants; development of positive control material to guard against cross-contamination of a diagnostic sample with the test positive control and; update data on the prevalence of GAV in *P. monodon* in NQ and JBG. In addition, determining the current status of enzootic viruses (e.g. HPV, MBV, MoV and SMV) in the sampled prawns was considered important and worthwhile value-adding to the project. Late in the process of drafting the TRF project proposal, requests were made from key APFA members to include an objective to also test samples for exotic viruses (WSSV and IMNV) of concern to them.

Objectives

1. Determine what GAV/YHV genotypes exist and their relative prevalence in wild *P. monodon* populations in NT/WA/QLD from which broodstock are captured for aquaculture purposes.
2. Revise PCR test designs as necessary to ensure their specificity, particularly in discriminating the highly virulent YHV1 strain that emerged in Thailand in the early 1990s from GAV and the other known YHV genotypic variants that appear to be far less pathogenic, and make these tests available for publication in the OIE Diagnostic Manual chapter for yellow head disease.
3. Acquire and/or generate appropriate control nucleic acids specific to the various YHV genotypes for use in YHV1 or other genotype-specific PCR tests so that their diagnostic specificity can be validated at key diagnostic laboratories (e.g., CSIRO-AAHL), and so that they can be made available to state and international laboratories with needs for equivalent diagnostic capabilities.
4. Determine the existence and prevalence of other enzootic viruses [e.g., Mourilyan virus (MoV), Monodon baculovirus (MBV), Hepatopancreatic parvovirus (HPV), Spawner-isolated mortality virus (SMV) and Infectious hypodermal and haematopoietic necrosis virus (IHHNV)] in wild *P. monodon* populations in the NT and QLD. In addition, test samples for the exotic viruses WSSV and IMNV given some broodstock are sourced from waters with a higher than usual likelihood of incursion from these pathogens.

Methods

Methods used at CSIRO-QBP

Penaeus monodon sampling locations and methods

Tissues were collected from *P. monodon* during mid to late 2013, with a few collected in early 2014. Tissues included gill, pleopod and hepatopancreas. Tissue pieces were either snap frozen on dry ice and stored at -80°C or preserved in RNAlater® solution (Life Technologies) at -20°C.

To obtain many of the samples, tissue sampling instructions, instruments and tubes containing RNAlater solution were provided to CSIRO staff participating in survey trawls in the GoC and to managers of commercial prawn hatcheries. In the case of hatcheries, it was requested that prawns be sampled as soon as practical after receipt. Sampling at this time was done so that the prawns were as representative of their wild counterparts as possible. As broodstock could not be sacrificed to collect tissue samples, digestive tract tissues such as hepatopancreas were not collected. In addition, samples had to be collected during the time window when hatcheries were operating and had broodstock. Due to this constraint, samples were collected mostly in mid to late 2013 in anticipation that the project proposal would be approved and prior to the project objectives being finalized. Late in the project application process the APFA requested inclusion of an additional objective to also test samples for selected enzootic (i.e. MoV, IHHNV, SMV, MBV and HPV) and exotic (i.e. WSSV and IMNV) viruses in addition to GAV/YHV. PCR testing to fulfil this objective was undertaken knowing that prevalence data, particularly for those viruses replicating primarily in digestive tract tissues (i.e. SMV, MBV and HPV), might be compromised by the lack of hepatopancreas (HP) or other more appropriate tissue types to test. As many of the prawns were sampled directly on trawlers or in commercial hatcheries, and as it was not possible to sacrifice broodstock in use at hatcheries, tissue types collected could not always include hepatopancreas.

Adult *P. monodon* were sourced from 3 disparate regions in northern Australia. These regions were selected as they encompass the primary locations where *P. monodon* broodstock occur in relative abundance and are either currently being captured for use in hatcheries or have been sourced previously as founder stocks for domestication and selective breeding programs. The 3 regions were:

1. Shallow inshore waters spanning the NQ region from Yorkeys Knob/Taylor Beach north of Cairns to Etty Bay south of Innisfail. The *P. monodon* (broodstock quality) sampled from this region were primarily supplied directly and generously free-of-charge from a commercial broodstock collector. As soon as practical after capture, prawns were air freighted alive from Cairns to Brisbane. Sampling of prawns directly after capture avoided possible cross-infections that might occur during their extended use at a hatchery. Samples were also collected from a batch of NQ prawns after their use at a hatchery in south-east QLD and some 6-8 weeks after their capture.
2. Unspecified trawl locations in JBG spanning the Northern Territory (NT) and Western Australia border and obtained either pre- or post-spawning from 3 QLD hatcheries that commissioned their capture. All prawns were sampled at the hatchery by hatchery staff and except for one batch that was sampled post-spawning (6-8 weeks after receipt), were sampled within 3-8 days of receipt.

3. Eastern and southern locations on the QLD side and western locations on the NT side of the GoC trawled at 6 monthly intervals (Feb and Jun 2013, Jan 2014) by CSIRO survey trawlers commissioned to assess prawn abundance and makeup to set seasonal fishing quotas.

Details of capture locations, sampling dates, prawn numbers and histories in hatcheries including tissue types collected and whether prawns were sampled pre- or post-spawning are summarized in **Figure 1** and **Table 1**. Tissues were obtained from >150 individuals originating from the JBG NQ locations, which would allow virus detection down to a 2% infection prevalence using a highly sensitive and specific diagnostic test. GoC prawns were sampled opportunistically by CSIRO staff participating in short survey trawls at predetermined trawl locations commissioned by the Australian Fisheries Management Authority to set seasonal trawl time allocations for the Northern Prawn Fishery. However, due to the nature of these trawls and a relative scarcity of *P. monodon*, samples were only obtained for 67 prawns over 3 surveys between February 2013 and February 2014.

Due to the large numbers of prawns sampled, only representative samples from NQ and JBG were tested for GAV/YHV. The sample sets were sourced from different hatcheries at different time points and different locations within a region. Sample numbers tested for other viruses were determined based on up to approximately 150 prawns per region, the availability of DNA samples from appropriate tissues and specific requirements from some hatcheries that samples could only be provided on the condition that they not be tested for exotic viruses such as WSSV. For these reasons, not all samples collected for potential use in the project and listed in **Table 1** were tested.

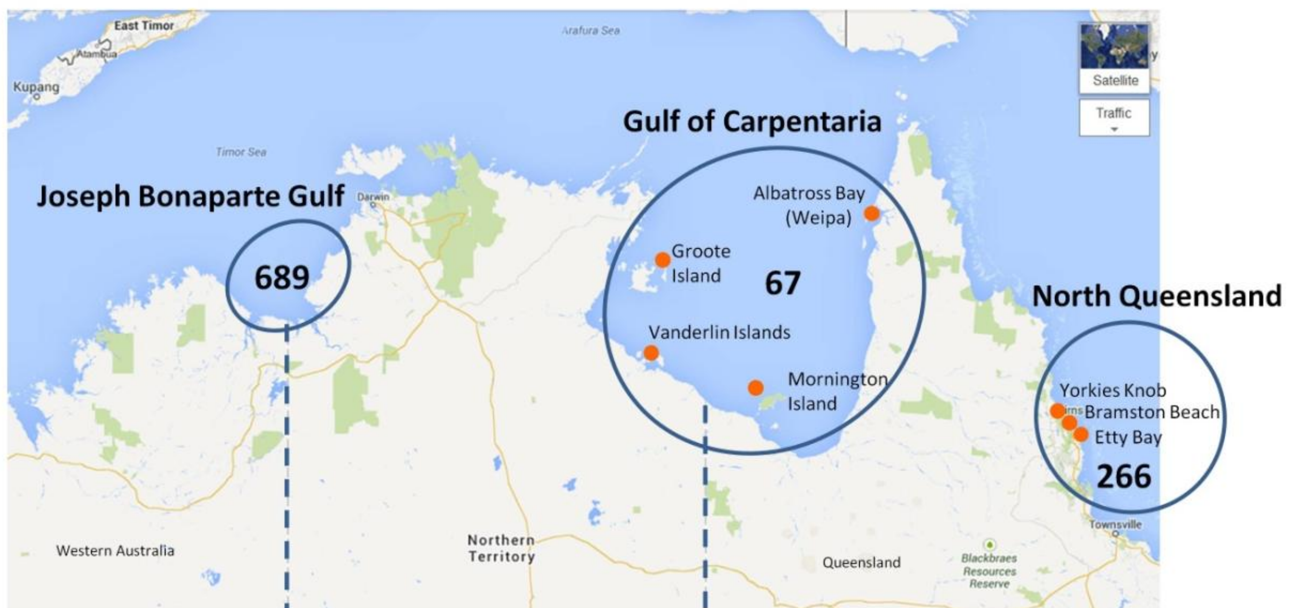

Figure 1. Sample numbers and general locations from where *P. monodon* broodstock were collected.

Table 1. Prawn numbers, collection locations and dates, suppliers and tissue types selected to extract RNA and DNA for PCR analysis

Region	Location	Date	Supplier	When sampled after arrival at the hatchery	Number	Gill/pleopod RNA	Gill/pleopod DNA	Hepatopancreas DNA
JBG	NK	13-06-2013	QLD Hatchery 1	6-8 wks (~4 wks post-spawn)	20	20		20 (4 tested)
	NK	04-08-2013	QLD Hatchery 2	8 days (pre-spawn)	175	150	120	
	NK	23-08-2013	QLD Hatchery 2	3 days (pre-spawn)	35	35	16	
	NK	05-09-2013	QLD Hatchery 3	3 days (pre-spawn)	239	40		
	NK	11-10-2013	QLD Hatchery 1	~3 days (26) post-spawn (20)	26*	26 (pre-spawn)		20 (post-spawn) (3 tested)
	NK	27-11-2013	QLD Hatchery 2	No information	194			
			Total=		**689**	**271**	**136**	**40 (7 DNA samples tested)**
GoC	Weipa	02-2013	CSIRO Trawler	Direct from wild	20	20	17	
	Vanderlin	02-2013	CSIRO Trawler	Direct from wild	20	20	6	
	Groote	02-2013	CSIRO Trawler	Direct from wild	1	1	1	
	Karumba - east	02-2013	CSIRO Trawler	Direct from wild	3	3	3	
	Karumba - west	02-2013	CSIRO Trawler	Direct from wild	3	3	3	
	Mornington - west	02-2013	CSIRO Trawler	Direct from wild	3	3	4	
	Vanderlin - east	02-2013	CSIRO Trawler	Direct from wild	2	2	2	
	Groote - south	02-2013	CSIRO Trawler	Direct from wild	2	2	2	
	Mornington - west	06-2013	CSIRO Trawler	Direct from wild	1	1	1	1
	Vanderlin - west	06-2013	CSIRO Trawler	Direct from wild	4	4	4	4
	Groote - south	06-2013	CSIRO Trawler	Direct from wild	2	2	2	2
	Groote - north	06-2013	CSIRO Trawler	Direct from wild	2	2	2	2
	Vanderlin - west	01-02-2014	CSIRO Trawler	Direct from wild	4	4	4	4
			Total=		**67**	**67**	**50**	**13**
NQ	Bramston Beach	24-06-2013	Trawler Operator 1	Direct from wild	51	51	51	51 (not tested)
	Yorkeys Knob-Taylor Beach	15-07-2013	QLD Hatchery 3 - Trawler Operator 2	No specific information	115	44		
	Etty Bay	18-07-2013	Trawler Operator 1	Direct from wild	60	60	60	60
	Bramston Beach	11-11-2013	Trawler Operator 1	Direct from wild	40		40	
			Total=		**266**	**155**	**151**	**111 (60 DNA samples tested)**

* gill/pleopod sampled from 26 prawns ~3 days after arrival at the hatchery, 20 of the same prawns remaining alive were sampled again after spawning; NK= not known; JBG= Joseph Bonaparte Gulf; GoC= Gulf of Carpentaria

Nucleic acid extraction, cDNA synthesis, PCR and DNA sequencing methods

RNA extraction

Where gill was available, it was used preferentially. Pleopod was used only in cases where samples had been collected on trawlers surveying prawn numbers at locations in the GoC and non-sacrificially from valuable *P. monodon* broodstock in use at commercial hatcheries. A small piece of prawn tissue was removed from -80°C or RNALater™, blotted briefly on paper towel to remove excess liquid and placed in a tube containing 0.5 ml RLT buffer (QIAGEN). The tissues was disrupted by 2 x 20 s cycles of bead beating using a PowerLyzer® bead beater (MO BIO), and total RNA was then extracted in 96-well plates using reagents and the protocol supplied in the RNeasy® 96 kit (QIAGEN). RNA concentration and quality were estimated by determining A_{260nm}/A_{280nm} using a Nanodrop ND8000 spectrophotometer, and RNA aliquots were normalised to 100-125 ng/µl using RNase-free water. RNA was generally converted into complementary DNA (cDNA) on the same day, with remaining RNA stored at -80°C for subsequent analysis.

DNA extraction

A small piece of prawn tissue (gill, pleopod or HP) was removed from RNALater™, blotted briefly on paper towel to remove excess liquid and digested in 180 µl Lysis Buffer and 20 µl Proteinase K (Thermo Scientific) overnight at 56°C. Total DNA was then extracted in 96-well plates using a Kingfisher Robot and MagJET Genomic DNA kit (Thermo Scientific) and eluted in 70 µl elution buffer. DNA concentration and quality were estimated by determining A_{260nm}/A_{280nm} using a Nanodrop ND8000 spectrophotometer and stored at -20°C until used. DNA was diluted either 1:5 or 1:10 in water prior to PCR to dilute PCR inhibitors when these were sometimes identified to reduce PCR amplification efficiency of host 18S ribosomal (r)DNA using generic primers for detection of decapod 18S ribosomal (r)DNA (Lo *et al.* 1996).

cDNA synthesis

Random-primed cDNA was synthesised using 4 µl (500 ng) total RNA, 1 µl (100 ng) random hexamer primers and cDNA synthesis reagents in the GoScript cDNA synthesis kit (Promega). RNA and primer (5 µl) were incubated at 70°C/5 min, GoScript reagents containing GoScript reverse transcriptase were then added (total vol of 10 µl) and the reaction incubated at 25°C/5 min, 42°C/1 h and 70°C/15 min according to the kit protocol. For IMNV which contains a dsRNA genome, RNA samples were heated at 95°C/5 min to strand-separate dsRNA before proceeding with cDNA synthesis. cDNA was stored at -20°C until used in PCR tests.

TaqMan real-time quantitative PCR

The TaqMan RT-qPCR tests for the ssRNA viruses GAV (de la Vega *et al.* 2004), YHV7 (JA Cowley *et al.* unpublished, described here) and MoV (Rajendran *et al.* 2006), the dsRNA virus IMNV (Andrade *et al.* 2007) and the TaqMan qPCR test for the dsDNA virus WSSV (East *et al.* 2004) employed 2 µl of either cDNA (~100 ng total RNA) or DNA, 900 nM each forward and reverse primer, 250 nM FAM-labelled TaqMan-probe and 1 x SensiFAST™ Probe Lo-ROX Mix (Bioline) in a 20 µl reaction volume. Aliquots (5 µl) of each PCR master mix were transferred into 3 wells of a 384-well PCR plate as technical replicates, and real-time qPCR thermal cycling and data capture utilized a ViiA7™ Real-time qPCR System (Life Technologies). PCR primer sequences and thermal cycling conditions used in these TaqMan RT-qPCR tests are detailed in **Appendix 1**.

Conventional PCR tests

The nPCR test used to detect MBV and the semi-nPCR test used to detect HPV were designed in-house at CSIRO-QBP in 2011 (S. Waqairatu and J.A. Cowley, unpublished). Each test utilized PCR primers designed to amino acid sequences conserved among different genotypes of each virus for which sequences had been published and/or were available in GenBank. The PCR primers incorporated redundancy to accommodate sequence variation among genotypes with the aim of maximizing their ability to amplify all known genotypes as well as potentially unknown genotypes.

SMV was detected using the nPCR test reported by Owens and Cullen (2004).

The PCR test used to detect IHHNV was that employing the 309F1 and 309R1 primers designed to only detect IHHNV and not the non-infectious IHHNV-like homologues integrated in the genome of a high percentage of Australian *P. monodon* (Tang *et al.* 2007).

The nPCR test used to detect any YHV genotype employed slightly modified thermal cycling conditions and primer sequences of the consensus YH30F/YH30R PCR (i.e., YH30Fm/YH30Rm) and YH31F/31R nPCR (i.e., YH31Fm/YH31Rm) described previously (Wijegoonawardane *et al.* 2008a). These modifications were made to broaden the potential specificity of the test for new/unknown YHV genotypes.

To identify DNA products amplified by these tests, an aliquot (7 µl) of each reaction was resolved by electrophoresis in a 2% agarose gel containing 0.5 µg/ml ethidium bromide, with DNA bands detected and photographed using a Gel Doc 2000 UV transilluminator (BioRad).

For representative DNA samples extracted from gill or HP tissue the 18S rDNA PCR test (Lo *et al.* 1996) was performed to confirm nucleic acid integrity.

In general, PCR tests used the 10 x PCR buffer and 50 mM $MgCl_2$ provided with the native *Taq* DNA polymerase (Invitrogen) used to amplify DNA. The 10 mM dNTP (dATP, dTTP, dCTP, dGTP) mixture used was also purchased from Invitrogen. PCR primer sequences and thermal cycling conditions used in these conventional PCR tests are detailed in **Appendix 1**.

DNA Sequencing

To clean up PCR products before sequencing, an aliquot (2 µl) of each PCR was treated at 37°C for 30 min with 2.5 U Exonuclease I (BioLab) to degrade residual single-stranded oligonucleotide primers and with Prawn Alkaline Phosphatase (SAP, BioLab) for removal of dNTPs, followed by incubation at 95°C for 5 min to inactivate the enzymes. Following this ExoSAP process, the treated DNA was sequenced in 20 µl reactions containing 3.2 pmol of either the forward or reverse primer and BDT3.1 BigDye® Direct Cycle Sequencing Kit reagent (Life Technologies) using the default thermal cycling conditions. The DNA was purified using the Agencourt® CleanSEQ (Beckman Coulter) dye-terminator removal method and samples were then transferred into a 96-well plate for capillary electrophoresis using an Applied Biosystems 3130XL Genetic Analyzer.

Methods used at AFDL

Sample processing and nucleic acid extraction

Fresh, frozen, ethanol-fixed or RNAlater-preserved *P. monodon* tissue was homogenised in 600 µl AVL buffer in MagNA Lyser Green Bead tubes using the MagNA Lyser (Roche) and clarified by centrifugation at 10,000 x *g* for 5 min. Total nucleic acid (TNA) was extracted from 140 µl clarified lysate using the QIAmp Viral RNA Mini Kit (QIAGEN) and eluted in 60 µl elution buffer.

PCR tests

Real-time (TaqMan) RT-qPCR tests were performed in 25 µl reactions containing 2 µl TNA, 12.5 µl 2 x AgPath-ID one-step RT-PCR buffer, 1 µl 25 x RT-PCR enzyme mix (Life Technologies), 900 nM each primer and 250 nM TaqMan probe. Tests were performed in a 7500 Fast Real-time qPCR System (Life Technologies) using the default thermal cycling conditions and analysed using the 7500 software. Samples were tested in duplicate with each generating a typical amplification curve to be classified as positive. A relevant positive control and a no template control were included in each test plate. PCR primer sequences and thermal cycling conditions used in each RT-qPCR test are detailed in **Appendix 2**.

Conventional RT-PCR tests were performed in 25 µl reactions containing 2 µl TNA, 12.5 µl 2 x reaction mix, 1 µl Superscript® III RT/Platinum® *Taq* mix (Life Technologies), 180 nM each primer and molecular grade water. For each nPCR, 2 µl of the RT-PCR were added to a 23 µl reaction mix containing 12.5 µl HotStar*Taq* master mix (QIAGEN), 360 nM each primer and molecular grade water. A relevant positive control and a no template control were included in each test. Conventional PCR tests were performed in singlicate. PCR primer sequences and thermal cycling conditions used in each conventional PCR test are detailed in **Appendix 2**.

Sequencing

DNA amplified in the conventional PCR tests was visualised after electrophoresis in SYBR Safe-stained 1.5% agarose gels. DNA to be sequenced was excised from the gel and purified using a QIAquick gel extraction kit (QIAGEN). DNA was Sanger-sequenced using both the forward and reverse primers, BigDye® Terminator v3.1 Cycle Sequencing reagent and the default cycle sequencing conditions in either a 3130xl or a 3500xl Genetic Analyzer (Life Technologies).

Phylogenetic analysis

Geneious software (Biomatters) was used to generate consensus sequences, multiple sequence alignments and to calculate pair-wise distances. MEGA5.1 software was used to undertake phylogenetic analyses using the neighbour-joining method together with a bootstrap consensus tree inferred from 10,000 replicates (Tamura *et al.* 2011).

Results and Discussion

Objective 1: Determine what GAV/YHV genotypes exist and their relative prevalence in wild P. monodon populations in NT/WA/QLD from which broodstock are captured for aquaculture purposes

PCR primers and probes and thermal cycling conditions used for the TaqMan real-time qPCR and conventional PCR tests for **Objective 1** conducted at QBP are described in **Appendix 1** and those conducted at AFDL are described in **Appendix 2**.

Prevalence of Gill-associated virus (GAV) / Yellow head virus (YHV) genotypes

The prevalence of GAV/YHV genotypes in *P. monodon* inhabiting the 3 northern Australian study regions was determined using RNA extracted from either gill or pleopod tissue and 3 PCR tests. The PCR tests used for screening and to amplify and sequence PCR products to confirm which YHV genotype had been amplified were:

1. The TaqMan real-time RT-qPCR test used routinely to detect GAV (de la Vega *et al.* 2004), which is known to be enzootic in *P. monodon* inhabiting the east coast of QLD.

2. A new TaqMan real-time RT-qPCR test designed as part of this project to specifically detect YHV7 as described in **Objective 2**. This YHV7 genotype was recently detected in *P. monodon* broodstock originating from JBG (Mohr *et al.* 2015).

3. A revision of the YH30/YH31 RT-nPCR test used to assign YHV genotypes in an extensive regional study (Wijegoonawardane *et al.* 2008a) employing modified PCR primers (YH30-F1m/R1m) and nPCR primers (YH31-F2m/R2m) that included slightly increased redundancy with the aim of broadening their specificity for amplifying unknown YHV genotypes (**Appendix 2**).

GAV

For 493 *P. monodon* originating from locations in NQ (n = 155), GoC (n = 67) and JBG (n = 271), RNA was extracted from gill/pleopod tissue at QBP, quantified and used to prepare cDNA. This cDNA was then tested at QBP using the GAV TaqMan real-time qPCR test. A much larger number (n = 271) of JBG prawns than specified (n = 150) was tested to ensure representative coverage of different cohorts sampled from different hatcheries at different times. Increasing the number of batches tested also provided potentially better coverage of the JBG study region as exact locations where the prawns were trawled were not provided by the hatcheries.

In total, 195 (39%) *P. monodon* tested positive using the GAV TaqMan RT-qPCR with C_T values ranging from 25 to 39. Of these, 9 had a C_T<30, 54 had a C_T value between 30 and 35 and 132 had a C_T value >35 (**Table 2**). The test was positive for 27% of the 271 *P. monodon* originating from JBG and sampled from the 3 QLD hatcheries. The highest prevalence of test positives (98%) was recorded at Hatchery 1 for the batches of 20 and 26 prawns sampled in June and October 2013, respectively. Of the samples generating C_T values <30, all were from JBG sourced prawns sampled from the QLD hatcheries. Among the 67 *P. monodon* sampled from GoC locations, 16% tested positive and among

the 155 prawns sampled from NQ locations, 72% tested positive with the highest prevalence (93%) detected for the group of 60 prawns trawled at Etty Bay.

Aliquots of the same cDNA samples tested using the GAV TaqMan RT-qPCR were also tested at QBP using the YHV7 TaqMan RT-qPCR developed in **Objective 2**. Using this test, 29 (6%) of the 493 samples tested positive for YHV7 with C_T values ranging from 20 to 37 (**Table 2**). Of these, 5 had a C_T value <30, 4 had a C_T value in the >30/<35 range and 20 had a C_T value >35. Of the 271 *P. monodon* originating from JBG and sampled from the QLD hatcheries, 25 (9%) were YHV7 positive, with these originating from Hatchery 1 (n = 18) and Hatchery 2 (n = 7) (**Table 2**). Positives with C_T values <25 were detected among samples from both Hatchery 1 and Hatchery 2. Only 3/155 NQ samples tested positive, 2 from Etty Bay and 1 from Yorkeys Knob. One sample collected at Weipa also tested positive among the 67 examined from GoC locations. All 4 of these samples tested positive at C_T values >35.

Table 2. GAV and YHV7 detection by TaqMan RT-qPCR in *P. monodon* samples collected from northern Australia

		GAV TaqMan qPCR	**YHV7 TaqMan qPCR**
	No. Samples	**No. Positive**	**No. Positive**
JBG			
QLD Hatchery 1	46	45 (98%)	18 (39%)
QLD Hatchery 2	185	18 (10%)	7 (4%)
QLD Hatchery 3	40	10 (25%)	0
JBG Total =	271	73 (27%)	25 (9%)
GoC			
Groote Island	8	1 (13%)	0
Vanderlin Islands	29	7 (24%)	0
Mornington Island	4	0	0
Weipa	20	2 (10%)	1 (5%)
Karumba	6	1 (17%)	0
GoC Total =	67	11 (16%)	1 (1%)
NQ			
Bramston Beach	51	41 (80%)	0
Yorkeys Knob-Taylor Point	44	14 (32%)	1 (2%)
Etty Bay	60	56 (93%)	2 (3%)
NQ Total =	155	111 (72%)	3 (2%)

The YH30m/YH31m nPCR was used at QBP to test all 493 *P. monodon* cDNA samples as a means of confirming the GAV and YHV7 TaqMan RT-qPCR data and possibly identifying any other YHV genotypes that might be present in the prawns. Analysis of an aliquot of each YH31m nPCR by electrophoresis in ethidium bromide-stained agarose gels identified an amplicon of the expected size for 97 of the cDNA samples. All of these samples were also positive using either the GAV or the YHV7 TaqMan RT-qPCR. With regard to the sample origin, YH31m nPCR-positives were identified for 44 *P. monodon* originating from JBG, 53 from NQ locations and none from GoC locations (**Table 3**). Positives were identified among batches of JBG *P. monodon* sampled from each of the 3 NQ hatcheries and among *P. monodon* originating from each of the 3 NQ study locations.

Table 3. Summary of YH30m/YH31m nPCR positives and the genotype of each by sequence analysis

Origin	Number tested	Number Positive	GAV Sequence	YHV6 Sequence	YHV7 Sequence
JBG					
QLD Hatchery 1	46	30*	26	0	3
QLD Hatchery 2	185	11	10	0	1
QLD Hatchery 3	40	3	3	0	0
GOC					
All regions sampled	67	0	0	0	0
NQ					
Bramston Beach	51	20	20	0	0
Yorkeys Knob	44	4	4	0	0
Etty Bay	60	29	22	7	0
Total =	493	97	85	7	4

* One amplicon not sequenced due to insufficient product

Of the 96 YH31m nPCR positives that were sequenced, 86 generated chromatograms of sufficient quality to generate a ~500 bp contig internal to the YH31m nPCR primers. Phylogenetic analysis of these 86 sequences identified 3 distinct clusters. Blastn analysis and pairwise comparison of sequences from each cluster with reference sequences for YHV1 to YHV10 (excluding YHV9 for which no sequence in this ORF1b gene region is currently available) determined that the sequence clusters represented genotypes GAV (YHV2), YHV6 and YHV7 (**Table 3**). Shorter contigs generated for the other 10 YH31m nPCR positives were sufficient to determine that they also clustered within these 3 genotypes. Thus for all 96 positives, 85 clustered with GAV, 7 clustered with YHV6 and 4 clustered with YHV7. GAV was detected in the batches of JBG prawns sampled from each of the 3 QLD hatcheries and in prawn batches sampled from each of the 3 NQ locations examined. Etty Bay was the only NQ location to have *P. monodon* in which both GAV and YHV6 were detected. Prawns in which a YHV7 sequence type was detected were restricted to those originating from JBG and sampled from 2 of the 3 QLD hatcheries. All 4 prawns in which a YHV7 sequence was identified were among those that tested positive using the YHV7 TaqMan RT-qPCR. While the YH30m/YH31m nPCR test is capable of amplifying all YHV1 to YHV10 genotypes (as demonstrated in **Objective 2,** YHV9 has not yet been tested), the low number of YHV7 sequences identified among the YH31m nPCR products amplified from JBG prawns sampled from the QLD hatcheries is likely to reflect a preference of the test primers for the GAV sequence in samples in which both GAV and YHV7 occur.

The GAV nucleotide sequences were all highly similar, with identity ranging from 97.4% to 100% despite them coming from *P. monodon* originating from different regions. The YHV6 sequences detected in Etty Bay prawns ranged in nucleotide identity from 98.6% to 100% and the YHV7 sequences detected in JBG prawns sampled from the 2 QLD hatcheries ranged in nucleotide identity from 98.2% to 100%. The GAV and YHV6 sequences were quite similar to each other (95.5% to 97.6% identity) and each of these genotypes possessed similar identity levels to YHV7 (i.e. 82.5% to 84.6% identity for GAV and 82.5% to 83.7% identity for YHV6).

To examine the relationships of the GAV, YHV6 and YHV7 sequences to reference sequences of representative YHV1 to YHV10 genotypes (excluding the YHV9 sequence which is not available), the 492 nt YH31m amplicon region of the ORF1b gene was used to generate a phylogenetic tree (**Figure 2**). Due to the large number of highly similar GAV sequences, only 6 from JBG prawns (2 from each QLD hatchery) and 2 from each NQ location were included in the analysis.

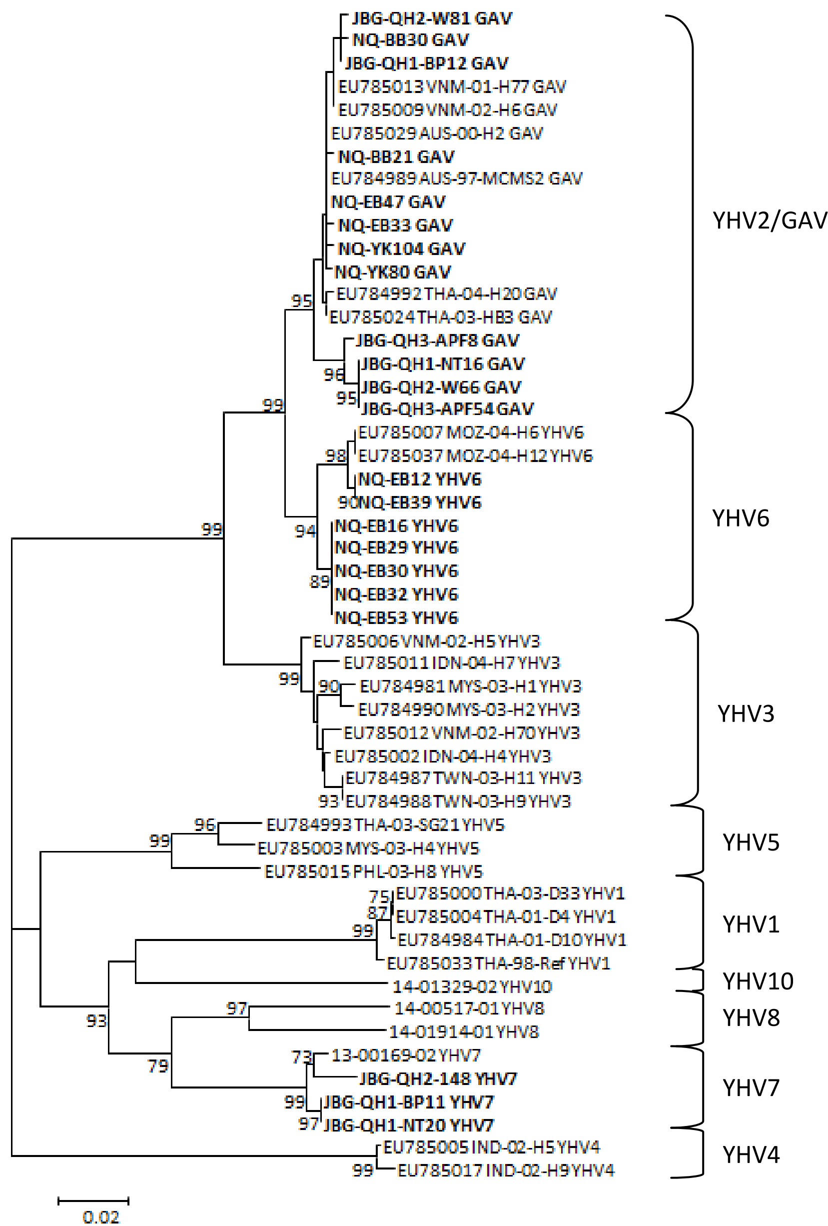

Figure 2. An unrooted neighbour-joining distance tree based on 492 bp ORF1b (YH31m nPCR) for representatives of YHV1 to YHV10 (YHV9 not available) and TRF samples (highlighted in bold).The phylogenetic analysis was based on 10,000 boot-strap replicates and only bootstrap-support >70% are shown on the tree. The scale bar represents substitutions per nucleotide site.

Table 4. Additional PCR testing undertaken on the 29 *P. monodon* that tested positive using the YHV7 TaqMan RT-qPCR

Sample Id	YHV7 qPCR C_T mean	GAV qPCR C_T mean	YH30m/YH31m nPCR	YH31m Sequence	YHV7 nPCR
JBG-QH1-BP9	22.0	31.9	+	GAV	+
JBG-QH1-BP10	36.9	31.2	+	GAV	-
JBG-QH1-BP11	29.9	34.4	+	YHV7	+
JBG-QH1-BP12	28.2	32.3	+	GAV	+
JBG-QH1-BP13	34.3	35.2	+	YHV7	+
JBG-QH1-BP14	33.4	35.1	-	-	-
JBG-QH1-BP15	31.1	32.5	+	GAV	+
JBG-QH1-BP16	35.4	36.6	+	NA*	+
JBG-QH1-BP17	36.9	34.5	+	GAV	+
JBG-QH1-BP18	35.1	32.2	+	GAV	-
JBG-QH1-BP20	37.0	UD	-	-	-
JBG-QH1-NT19	36.6	31.3	+	GAV	+
JBG-QH1-NT20	23.0	34.9	+	YHV7	+
JBG-QH1-NT21	37.0	24.8	+	GAV	-
JBG-QH1-NT22	36.7	34.6	+	GAV	-
JBG-QH1-NT23	37.1	36.5	-	-	-
JBG-QH1-NT24	35.7	35.4	-	-	+
JBG-QH1-NT25	36.1	36.6	-	-	-
JBG-QH2-W33	36.8	UD	-	-	-
JBG-QH2-W37	35.8	UD	-	-	-
JBG-QH2-W57	37.5	UD	-	-	-
JBG-QH2-147	35.8	37.3	-	-	-
JBG-QH2-148	20.0	UD	+	YHV7	+
JBG-QH2-149	34.4	30.4	+	GAV	-
JBG-QH2-151	37.4	UD	-	-	+
GoC-WEI11	35.7	38.2	-	-	-
NQ-EB17	37.0	37.0	-	-	-
NQ-EB34	36.6	36.4	-	-	-
NQ-YK14	35.9	UD	-	-	-

NA* – not assessed due to insufficient YH31m nPCR amplicon for sequencing
UD= Undetermined, NQ= North Queensland, QH= Queensland Hatchery

Of the 111 samples that were either YHV7 TaqMan RT-qPCR (n = 29) or YH31m nPCR (n = 97) positive, only 4 of the 96 YH31m amplicons sequenced were identified to be YHV7. The YHV7 nPCR test (developed in **Objective 2**) was therefore performed on these 111 samples. Only 12 YHV7 nPCR positives were obtained, but none were sequenced. Each of these was also YHV7 TaqMan RT-qPCR positive (with C_T values <37), and all were from JBG *P. monodon* sampled from either QLD Hatchery 1 or Hatchery 2 (**Table 4**). Despite the additional level of confirmation provided by use of the YHV7 nPCR, the 4 YHV7 TaqMan RT-qPCR test positives from GoC and NQ (C_T values ranging from 35 to 37) were not confirmed by the YHV7 nPCR test. It is not unusual for TaqMan real-time qPCR positives with C_T values >35 to be problematic, due to low levels of target, to confirm by conventional PCR.

Therefore, YHV7 has only been confirmed to occur in *P. monodon* originating from the JBG and sampled from QLD hatcheries.

Interestingly, 76% (n = 22) of the YHV7 TaqMan RT-qPCR positive *P. monodon* were also GAV TaqMan RT-qPCR positive (**Table 4**). Of these, 10 were YH31m nPCR positive with the sequence identified as GAV. The data suggest that many of the YHV7-positive *P. monodon* were also infected with GAV. To investigate this finding further, total nucleic acid (TNA) from sample JBG-QH2-149 (GAV and YHV7 qPCR positive) was tested at AFDL using the OIE YHV Protocol 3 RT-nPCR and YHV7 RT-nPCR. Both generated nPCR amplicons of the expected size with each test. Sequence analysis of the OIE YHV Protocol 3 RT-nPCR amplicon and YHV7 RT-nPCR amplicon identified GAV and YHV7, respectively. Therefore it is likely that the *P. monodon* that tested positive in both the YHV7 and GAV TaqMan RT-qPCRs contained dual YHV7 and GAV infections.

The YHV6 genotype was identified as a distinct genetic lineage by Wijegoonawardane *et al.* (2008a) based on its detection only in healthy *P. monodon* reported as originating from Mozambique and provided among a batch of tissue samples from broodstock being cultured at a commercial breeding facility in Malaysia during 2004. This phylogenetic distinction was made despite YHV6 being closely related to GAV when nucleotide sequences of the ORF1b region were compared. YHV6 and GAV were indistinguishable when the translated amino acid sequence over the ORF1b region was compared (Wijegoonawardane *et al.* 2008a). Since 2008, detection of YHV6 in *P. monodon* has not been further reported. It was therefore somewhat unexpected that YHV6 was identified in several *P. monodon* trawled from Etty Bay in NQ. Due to this discovery, QBP examined archived sequences of PCR products amplified using the GAV5/6 PCR test (Cowley *et al.* 2000) that was in common use to detect GAV prior to the development of the GAV TaqMan real-time qPCR test. Among 4 sequences amplified from *P. monodon* broodstock originating from either Weipa in the GoC or from an unspecified NQ location in 2002, 3 of the 4 PCR amplicons from the NQ prawns were identified to be YHV6 rather than GAV (**Figure 3**).Therefore, it is clear that YHV6 has existed together with GAV in NQ P. monodon since at least 2002. Due to the very high sequence similarity with GAV, it is not surprising that YHV6 is also amplified by the GAV TaqMan RT-qPCR.

In addition to the samples obtained by QBP for this project, QDAF provided AFDL with gill tissue from P. monodon sampled from 2 QLD hatcheries (coded QH4 and QH5 as their relationship to QLD Hatcheries 1 to 3 is unknown), over 5 submissions from 2013 to 2014. These samples were submitted to assist test development due to the discovery of YHV7 by AFDL. Permission to screen these samples for YHV genotypes was granted by QDAF. Samples from QLD Hatchery 4 were from broodstock imported from JBG (n = 9) and their F1 offspring reared at a farm in QLD (n = 11). Samples from QLD Hatchery 5 were from broodstock sourced from NQ (n = 14). Total nucleic acid (TNA) was extracted from the samples and tested using the YHV7 TaqMan RT-qPCR, YHV7 RT-nPCR and GAV TaqMan RT-qPCR (**Table 5**). All 20 broodstock/F1 samples from QLD Hatchery 4 were positive by the YHV7 TaqMan RT-qPCR (C_T values for broodstock ranged from 31 to 40 and for offspring ranged from 16 to 35). Of these samples, 18 were also YHV7 RT-nPCR positive, with YHV7 confirmed by sequencing of 2 amplicons each from broodstock and F1 offspring. Of the 14 QLD Hatchery 5 samples, 4 were YHV7 TaqMan RT-qPCR positive (C_T values ranged from 38 to 41). However, none of these samples were positive by YHV7 RT-nPCR. Therefore, the YHV7 TaqMan RT-qPCR generated positive results for NQ *P. monodon* which could not be confirmed using the

conventional YHV7 nPCR test. As stated previously, it is not uncommon for samples generating real-time qPCR positive results with C_T values >35 being negative by conventional PCR. The GAV TaqMan RT-qPCR test detected GAV in *P. monodon* from both hatcheries with C_T values ranging from 19 to 38. For samples AFDL-QH4-09 to 14, the OIE YHV/GAV Protocol 2 GAV nPCR was applied and confirmed the presence of GAV in 2 samples that had GAV TaqMan RT-qPCR C_T values of 26. This again provided evidence for the presence of dual YHV7 and GAV infections in broodstock in QLD hatcheries. The data from this testing has been shared with QDAF.

Figure 3. An unrooted neighbour-joining distance tree based on 497 bp ORF1b (YH31m nPCR) for representatives of Australian GAV and Mozambique YHV6, selected TRF samples (highlighted in bold) and QBP provided QLD 2002 sequences (highlighted in red). The phylogenetic analysis was based on 10,000 boot-strap replicates and only bootstrap-support >70% are shown on the tree. The scale bar represents substitutions per nucleotide site.

Table 5. GAV and YHV7 detection in *P. monodon* samples supplied to AFDL by QDAF

Sample ID	Origin	YHV7 RT-qPCR C_T mean	YHV7 nPCR	GAV RT-qPCR C_T mean
AFDL-QH4-01	JBG broodstock	31.0	+	35.7
AFDL-QH4-02	JBG broodstock	33.4	+	34.4
AFDL-QH4-03	JBG broodstock	36.0	+	33.4
AFDL-QH4-04	JBG broodstock	39.8	+	UD/38.0
AFDL-QH4-05	JBG broodstock	33.8	+	28.6
AFDL-QH4-06	JBG broodstock	33.2	+	32.8
AFDL-QH4-07	JBG broodstock	37.9	+	33.9
AFDL-QH4-08	JBG broodstock	33.8	-	32.1
AFDL-QH4-09	JBG broodstock - F1 offspring	21.8	+	37.8
AFDL-QH4-10	JBG broodstock - F1 offspring	19.1	+	34.9
AFDL-QH4-11	JBG broodstock - F1 offspring	19.6	+	26.2
AFDL-QH4-12	JBG broodstock - F1 offspring	17.9	+	31.0
AFDL-QH4-13	JBG broodstock - F1 offspring	17.7	+	25.5
AFDL-QH4-14	JBG broodstock - F1 offspring	15.7	+	33.0
AFDL-QH4-15	JBG broodstock - F1 offspring	34.5	+	20.8
AFDL-QH4-16	JBG broodstock - F1 offspring	34.3	+	22.9
AFDL-QH4-17	JBG broodstock - F1 offspring	34.1	+	18.8
AFDL-QH4-18	JBG broodstock - F1 offspring	32.9	+	23.3
AFDL-QH4-19	JBG broodstock - F1 offspring	34.3	-	18.6
AFDL-QH5-01	NQ broodstock	UD/42.7	-	33.6
AFDL-QH5-02	NQ broodstock	UD/36.3	-	31.0
AFDL-QH5-03	NQ broodstock	UD	-	29.8
AFDL-QH5-04	NQ broodstock	39.6	-	34.6
AFDL-QH5-05	NQ broodstock	38.2	-	32.3
AFDL-QH5-06	NQ broodstock	UD	-	33.8
AFDL-QH5-07	NQ broodstock	UD	-	UD
AFDL-QH5-08	NQ broodstock	UD	-	30.4
AFDL-QH5-09	NQ broodstock	40.3	-	32.0
AFDL-QH5-10	NQ broodstock	UD	-	35.6
AFDL-QH5-11	NQ broodstock	UD/37.3	-	28.9
AFDL-QH5-12	NQ broodstock	40.9	-	32.6
AFDL-QH5-13	NQ broodstock	UD	-	33.6
AFDL-QH5-14	NQ broodstock	UD/37.8	-	36.0

UD = Undetermined

To further investigate the possibility of YHV7 being present in QLD prior to it being identified in 2013, a sub-population of TNA extracted from *P. monodon* sampled from 7 farms in 2008 and submitted to AFDL for IHHNV screening was tested using the YHV7 TaqMan RT-qPCR. Permission to use the 2008 TNA samples for this purpose was granted by QDAF. The TNA samples from 6 of the 7 farms tested negative. As positives were detected in TNA from one farm, all available 161 TNA samples from that farm were tested. Of these, 13 were identified to be positive with C_T values ranging from 29 to 42. However, confirmatory testing using the YHV7 RT-nPCR resulted in no

positives. The analytical specificity (ASp) of the YHV7 TaqMan RT-qPCR as determined in **Objective 2** would suggest that only YHV7 was being detected. The analytical sensitivity (ASe) of the YHV7 RT-nPCR also determined in **Objective 2** would suggest that at least the 3 TNA samples with C_T values <30 should have been amplified by conventional nPCR. The lack of YHV7 RT-nPCR detection is likely due to the RNA quality having deteriorated after >7 years storage at -80°C and subjected to several freeze-thaw cycles during this period. As the YHV7 RT-qPCR targets a sequence <100 nt in length and the YHV7 RT-nPCR targets sequences >400 nt in length, the real-time qPCR test is less affected by RNA degradation. The data from this testing has been shared with QDAF.

Objective 2: Revise PCR test designs as necessary to ensure their specificity, particularly in discriminating the highly virulent YHV1 strain that emerged in Thailand in the early 1990's from GAV and the other known YHV genotypic variants that appear to be far less pathogenic, and make these tests available for publication in the OIE Diagnostic Manual for Yellowhead disease

All real-time TaqMan and conventional PCR tests for **Objective 2** were conducted at AFDL using the PCR primers and probes and thermal cycling conditions described in **Appendix 2**.

Analytical specificity of current yellow head complex PCR tests

The yellow head disease chapter of the OIE Manual of Diagnostic Tests for Aquatic Animals (OIE, 2014) currently recommends 3 conventional PCR protocols to detect and identify different genotypes within the YHV complex.

1. **OIE YHV Protocol 1 RT-PCR:** adapted from Wongteerasupaya *et al.* (1997) that can detect YHV1 in prawns affected by YHD (OIE, 2014). However, being only a single step RT-PCR, the test is less sensitive than RT-nPCR or RT-qPCR. In addition, the primer trimmed amplicon is only 94 nucleotides (nt) in length and thus of limited value in confirming the YHV1 genotype.

2. **OIE YHV/GAV Protocol 2 RT-nPCR:** adapted from Cowley *et al.* (2004) and is a more sensitive multiplex protocol than OIE YHV Protocol 1. The test was designed to differentiate YHV1 from GAV in diseased prawns or for screening healthy carriers (OIE, 2014). However, potential issues have been identified at AFDL with the specificity of the nPCR test employing the YHV1 Y3 primer. With GAV, the Y3 nPCR test generates a slightly longer amplicon which could be misinterpreted as YHV1.

3. **OIE YHV Protocol 3 RT-nPCR:** adapted from Wijegoonawardane *et al.* (2008b) and is a sensitive multiplex RT-nPCR protocol. This test can be used for screening healthy prawns for any of the 6 YHV genotypes, with genotype assignment achieved by nucleotide sequence analysis (OIE, 2014). YHV7 is also amplified by this test but in other investigations undertaken at AFDL, it has not been able to amplify YHV8 and YHV10. In addition, the primer-trimmed nPCR amplicon is only 94 nt in length and thus of limited value in confirming the YHV genotype.

Each of these 3 PCR tests target different regions of the ORF1b gene. The OIE YHV Protocol 1 RT-PCR test targets an ORF1b gene sequence 189 nt downstream of the sequence targeted by OIE YHV/GAV Protocol 2 RT-nPCR test, and the OIE YHV Protocol 3 RT-nPCR test targets a ORF1b sequence a further 3119 nt downstream of the OIE Protocol 1 RT-PCR test (**Figure 4A**).

To more fully assess the specificity of the 3 OIE YHV PCR test protocols for detecting YHV1 and other YHV genotypes, TNA prepared from clinical samples infected with YHV1, GAV, YHV7, YHV8 and YHV10 (available at AFDL) and additional RNA provided for GAV, YHV3, YHV4, YHV5 and YHV6 (available at CSIRO-QBP) was tested (**Table 6**). No nucleic acid was available for YHV9. PCR amplification and amplicon sequence analysis of nucleic acids from 2 samples of each genotype

(except YHV4, YHV6 and YHV10 for which only 1 sample was available) confirmed that each sample clustered with its designated genotype. The nucleic acids were then examined using each of the OIE YHV Protocol 1, 2 and 3 PCR tests.

Table 6. Description of each YHV genotype RNA sample in the YHV panel used for determining molecular test specificity

Isolate description	YHV Genotype	Source
Thailand - received 1998	1	AFDL
Thailand - received 2012	1	AFDL
Australia - experimental 2004/2008	2/GAV	QBP
Australia - sampled 2012	2/GAV	AFDL
Malaysia - MYS-03-H1	3	QBP
India - IDN-04-H4	3	QBP
Indonesia - IND-02-H5	4	QBP
Malaysia - MYS-03-H4	5	QBP
Philippines - PHL-03-H8	5	QBP
Australia (TRF) NQ EB39	6	QBP
Australia - sampled 2012	7	AFDL
Australia - sampled 2014	7	AFDL
China - sampled 2014	8	AFDL
China - sampled 2014	8	AFDL
China - sampled 2014	10	AFDL

Of the nucleic acid samples tested, the OIE YHV Protocol 1 RT-PCR test (**Figure 4B**) produced amplicons of the expected size with those containing strains of YHV1, and also produced just detectable amounts of an amplicon with those containing strains of YHV7 (**Table 7**). While amplified inefficiently, the YHV7 amplicon was confirmed to be YHV7 by sequence analysis. As the amplification of YHV7 by this test was not obvious in the initial discovery of this genotype at AFDL in 2013, its detection appears likely to be due to the relative amount of YHV7 RNA present in a sample. The reduced specificity for only YHV1 indicates a limited usefulness as a diagnostic tool without sequence analysis of amplicons to confirm the detection of YHV1 (and not YHV7). While only a single step RT-PCR, and not employing a nPCR step may reduce sensitivity, its relative sensitivity compared to other PCR tests specific for YHV1 has not been determined.

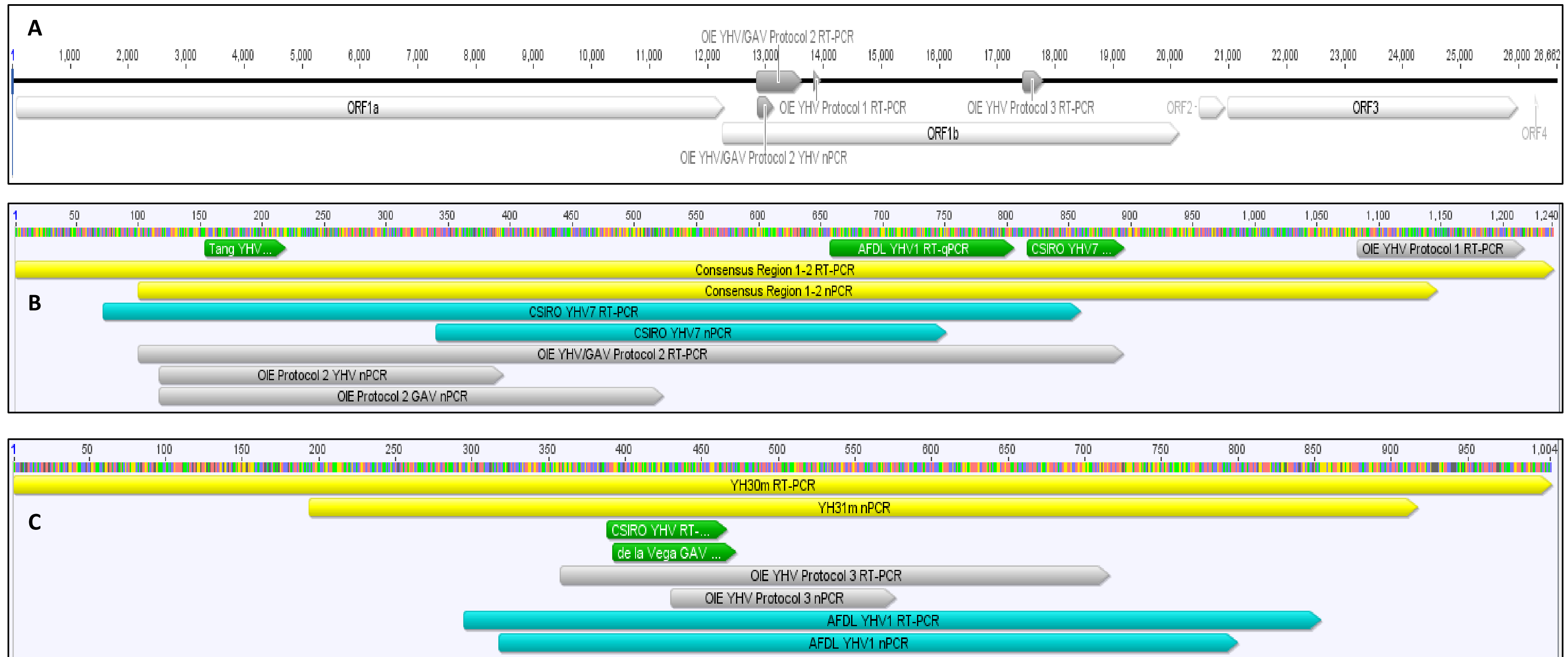

Figure 4. Genomic targets of YHV-complex real-time and conventinal RT-PCR assays. A) Location of the three OIE YHV PCR tests targeting ORF1b regions of the YHV1 genome (EU487200). B) Expanded OIE YHV Protocol 1 and 2 sequence region and C) Expanded OIE YHV Protocol 3 sequence region of ORF1b targeted by real-time (TaqMan) and conventional PCR tests used in **Objective 2**. Targeted regions for B) and C) were created based on the alignment of YHV1 (EU487200), GAV (AF227196) and YHV7 (13-00169-02). Highlighted in green are the regions targeted by RT-qPCRs.

Table 7. Analytical specificity (ASp) of the real-time (TaqMan) and conventional PCR tests tested in **Objective 2** with all available YHV genotypes. Expected positive (green) and other (yellow) detections for each test are highlighted.

	Real Time (TaqMan) qPCR					Conventional PCR						
Genotype	**CSIRO YHV RT-qPCR**	**Tang YHV RT-qPCR**	**de la Vega GAV RT-qPCR**	**AFDL YHV1 RT-qPCR★**	**CSIRO YHV7 RT-qPCR★**	**OIE YHV Protocol 1 RT-PCR**	**OIE YHV/GAV Protocol 2 RT-nPCR**	**OIE YHV Protocol 3 RT-nPCR**	**Consensus 1/2 RT-nPCR**	**YH30m/ YH31m RT-nPCR**	**AFDL YHV1 RT-nPCR★**	**CSIRO YHV7 RT-nPCR★**
YHV1	POS	POS	Neg	POS	Neg	POS	POS	POS	POS	POS	POS	Neg
YHV2/GAV	POS	POS	POS	Neg	Neg	Neg	POS*	POS	POS	POS	Neg	Neg
YHV3	Neg	Neg	POS	Neg	Neg	Neg	Neg	POS	POS	POS	Neg	Neg
YHV4	Neg	POS	POS	Neg	Neg	Neg	Neg	POS	POS	POS	Neg	Neg
YHV5	Neg	Neg	POS	Neg	Neg	Neg	Neg	POS	POS	POS	Neg	Neg
YHV6	Neg	Neg	POS	Neg	Neg	Neg	POS	POS	POS	POS	Neg	Neg
YHV7	Neg	POS	POS	Neg	POS	POS	POS^	POS	POS	POS	Neg	POS
YHV8	POS	Neg	Neg	Neg	Neg	Neg	Neg	POS[#]	POS	POS	Neg	Neg
YHV10	POS	Neg	Neg	Neg	Neg	Neg	Neg	Neg	POS	POS	Neg	Neg

* GAV amplification by both YHV1 nPCR and GAV nPCR

^ YHV7 amplification by the RT-PCR

[#] Only one of two YHV8 isolates amplified

★ New tests developed during the project

Pos = positive, Neg = negative

The OIE YHV/GAV Protocol 2 RT-nPCR test was performed by either running the YHV1 and GAV nPCR tests multiplexed together as described or running them separately (**Figure 4B**). In either case the tests amplified YHV1 and GAV as expected, and as found previously, the PCR step of the test also generated an amplicon for YHV7 (**Table 7**). The amplification of a GAV product slightly larger than the YHV1 product was again noted to occur in the YHV1 Y3 nPCR test. The GAV nPCR was observed to amplify the very closely related YHV6 genotype, which was confirmed by sequence analysis. Both the OIE YHV/GAV Protocol 2 RT-PCR and nPCR tests therefore lack specificity for their designed targets (e.g. YHV1 and GAV), limiting the use of the assay as a diagnostic tool unless the genotype detected is confirmed by sequence analysis of amplicons. For instance, in Australian *P. monodon* all three genotypes identified to be present in this project (GAV, YHV6 and YHV7) could be amplified and the exclusion of YHV1 could only be determined by sequencing. The yellow head disease Chapter in the OIE Manual of Diagnostic Tests for Aquatic Animals was updated in 2013 with a recommendation that all PCR products generated using the OIE YHV/GAV Protocol 2 RT-nPCR be sequenced to confirm the virus genotype.

The OIE YHV Protocol 3 RT-nPCR (**Figure 4C**) was able to amplify genotypes YHV1 to YHV7 as expected. However, a nPCR amplicon as verified by sequence analysis was only observed for 1 of the 2 YHV8 samples tested (**Table 7**). In addition, no nPCR amplicon was observed with the single YHV10 sample. Whether these negative results were due to test sensitivity or specificity limitations was not investigated and will require additional testing of more samples as well as sequence analysis of the YHV8 and YHV10 strains across the PCR primer binding sites. However, as the strains of YHV8 and YHV10 were not amplified consistently, and due to the relatively short (<100 nt) length of the primer-trimmed nPCR amplicon used to assign a genotype, the test should not be used in isolation for identifying new YHV genotypes.

Real-time qPCR tests are more commonly being used in diagnostic laboratories to provide rapid, highly sensitive and highly specific methods of detecting pathogen nucleic acid. For this reason they are routinely used as primary screening assays. While SYBR-Green qPCR tests are less expensive, qPCR tests using a TaqMan probe are generally more specific and reproducible. However, the yellow head disease Chapter of the OIE Manual of Diagnostic Tests for Aquatic Animals currently does not recommend any YHV real-time qPCR tests, despite the publication of several SYBR-Green and TaqMan-based real-time qPCR tests. Therefore, 3 different TaqMan real-time qPCR tests to detect either YHV or GAV were tested, using the panel of nucleic acids from the various YHV genotypes to examine the specificity of each assay.

The CSIRO YHV TaqMan RT-qPCR test (Jeff Cowley, CSIRO-QBP, unpublished) was designed to an ORF1b gene sequence within the OIE YHV Protocol 3 nPCR sequence (**Figure 4C**). The test amplified YHV1, but also cross-detected GAV, YHV8 and YHV10 (**Table 7**). Another YHV TaqMan RT-qPCR test described by Tang-Nelson and Lightner (2001) targeting a sequence within the region targeted by OIE YHV Protocols 1 and 2 (**Figure 4B**). This test detected YHV1 at a sensitivity comparable to the CSIRO YHV RT-qPCR, but also cross-detected GAV, YHV4 and YHV7 (**Table 7**). The GAV TaqMan RT-qPCR test described by de la Vega *et al.* (2004) targeted a sequence within the region targeted by the OIE YHV Protocol 3 nPCR (**Figure 4C**). This test detected GAV but also cross-detected YHV3, YHV4, YHV5, YHV6 and YHV7 (**Table 7**). While it was possible that YHV7 detections might have resulted from GAV being present as mixed infection in the same prawn, as found with samples of some JBG prawns tested to fulfil **Objective 1**, the presence of GAV was not confirmed by the OIE YHV/GAV Protocol 2 GAV nPCR test.

Based on these three published real-time TaqMan RT-qPCR tests for GAV/YHV each cross-detecting various genotypes other than GAV and YHV1, their capacity and limitations need to be considered in their application, particularly as identifying the YHV genotype is essential for diagnosis.

Design and validation of YHV1-specific PCR tests

As none of the OIE-recommended conventional PCR tests, published TaqMan real-time qPCR tests or the CSIRO YHV RT-qPCR test were determined to have adequate specificity for only the YHV1 genotype (which is highly virulent and exotic to Australia), new YHV1 TaqMan real-time RT-qPCR and conventional RT-nPCR tests were designed and evaluated. Based on an alignment of ORF1b sequences in the OIE YHV Protocol 1/2 region of YHV genotypes YHV1 to YHV10 (sequences from GenBank, AFDL and plasmids generated at QBP), PCR primers and a TaqMan probe were designed to sequences in which YHV1 was divergent from any of the other genotypes (**Figure 4B**). An alignment of ORF1b gene sequences in the OIE YHV Protocol 3 region for YHV genotypes YHV1 to YHV10 (excluding YHV9) were used to design PCR primers for use in a YHV1-specific conventional RT-nPCR test (**Figure 4C**).

The specificity of the newly-developed AFDL YHV1 TaqMan RT-qPCR and AFDL YHV1 RT-nPCR was evaluated using the YHV genotype panel (**Table 7**). Both tests detected each of the 2 YHV1 nucleic acid samples exclusively. The analytical specificity (ASp) was further evaluated with nucleic acids obtained from prawn tissue infected with other viruses including WSSV, IMNV and TSV (all exotic to Australia) as well as IHHNV, MoV, MBV, HPV and SMV (enzootic to Australia) (**Table 8**). None of these nucleic acids tested positive with either YHV1 PCR test.

Table 8. The origin and source of exotic and enzootic viruses in prawns used for molecular test specificity

Species (Country)	Virus	Nucleic Acid	Source
L. vannamei (China)	WSSV	DNA	AFDL
L. vannamei (Indonesia)	IMNV	RNA	AFDL
L. vannamei (Indonesia)	TSV	RNA	AFDL
L. vannamei (China)	IHHNV	DNA	AFDL
P. monodon (Australia)	MoV	RNA	QBP (this project)
P. monodon (Australia)	MBV	DNA	QBP (this project)
P. monodon (Australia)	HPV	DNA	QBP (this project)
P. monodon (Australia)	SMV	DNA	QBP (this project)

The analytical sensitivity (ASe) of the AFDL YHV1 RT-qPCR and YHV1 RT-nPCR tests was determined initially using YHV1 plasmid DNA (provided by QBP) diluted in water. The limits of detection of the AFDL YHV1 TaqMan RT-qPCR and YHV1 RT-nPCR tests were as low as 2 and 20 DNA copies per reaction, respectively. Based on the good ASp and ASe of the tests, a YHV1 dual-probe plasmid and a YHV1 artificial-insert plasmid were designed as controls for each test, respectively, to fulfil components of **Objective 3**. The ASe of the AFDL YHV1 TaqMan RT-qPCR was determined using the YHV1 dual-probe plasmid DNA control diluted either in water or prawn total nucleic acid. The limit of detection of the control DNA in either diluent was as low as 20 DNA copies per reaction (**Table 9**). The ASe of the AFDL YHV1 RT-nPCR test with the YHV1 artificial-insert plasmid DNA has yet to be determined. Synthetic RNA transcribed from either plasmid is still to be prepared and tested prior to distribution of the tests and positive controls.

Table 9. YHV1 TaqMan RT-qPCR test detection of the YHV1 dual-probe plasmid DNA diluted either in water or in prawn total nucleic acid

	YHV1 dual-probe plasmid DNA	
Copies/reaction	**Diluted in water (CT Mean)**	**Diluted in prawn TNA (CT Mean)**
200,000,000	7.2	7.7
20,000,000	12.7	12.0
2,000,000	15.9	15.8
200,000	19.5	19.5
20,000	22.9	23.4
2,000	27.5	27.5
200	32.8	31.1
20	35.8	37.5
2	UD	UD

UD = Undetermined

As the AFDL YHV1 TaqMan RT-qPCR was designed for inclusion in the OIE diagnostic manual, validation of the test according to OIE guidelines is required. Determining diagnostic sensitivity (DSe) is critical for test validation and varies depending on the primary test purpose (i.e. diagnosis vs surveillance). To determine the DSe of the AFDL YHV1 TaqMan RT-qPCR test, clinical samples known to be infected with YHV1 strains need to be sourced, as AFDL currently only has 4 isolates from Thailand and 1 isolate from Vietnam. Upon request, Dr Peter Walker (YHV OIE expert) provided nucleic acid for an additional Thai YHV1 strain. A further 5 nucleic acids of Thai YHV1 strains were provided by QBP. These 11 YHV1-positive samples are well short of the 100 desired for determination of DSe. As YHV1 is exotic to Australia, the search for YHV1-infected material or nucleic acid was broadened internationally. Requests from Dr Mark Crane (AFDL) and Dr Ingo Ernst (DoA) to several international laboratories in mid-2014 resulted in the receipt of only a single shipment comprising an additional 3 Thai YHV1 strains from Dr Chumporn Soowannayan, Mahidol University. At the 3rd OIE Global Conference on Aquatic Animal Health held in Ho Chi Minh City, Vietnam, 20-22 January 2015, Dr Nick Moody repeated this request for YHV1-positive material to delegates participating from international laboratories. At the submission of this final report, no additional offers of YHV1-positive material have been received. All 14 known-positive YHV1 samples were test positive with both the AFDL YHV1 TaqMan RT-qPCR and YHV1 RT-nPCR tests. The majority of YHV1 sequences submitted to GenBank have been generated from Thai isolates, making *in silico* analysis of any primer and/or probe mismatches for the newly designed PCR tests of limited value. As the virus has been reported to occur in other regions and prawn species, such as the Gulf of California and Pacific blue shrimp (*P. stylirostris*) (Castro-Longoria *et al.* 2008), any further genetic variation among YHV1 strains is unknown. As it seems unlikely that further YHV1-infected samples will be received, to further validate the 2 tests, the number of known-positive YHV1 samples will be increased by experimental infection of *P. monodon* in AFDL aquaria in the AAHL high-level biosecure facility as part of the FRDC Project 2015/005 investigating the pathogenicity of YHV7, YHV8 and YHV10 to Australian *P. monodon* and *P. merguiensis*. Prawns will be infected with YHV1 as positive infection controls during these trials.

To test the desired 300 known-negative *P. monodon* for determination of the diagnostic specificity (DSp) of both the AFDL YHV1 TaqMan RT-qPCR and YHV1 RT-nPCR, permission was granted by QDAF to use TNA prepared from samples submitted to AFDL in 2008 for IHHNV screening. Of the 74 TNA samples tested thus far using the AFDL YHV1 TaqMan RT-qPCR, all were negative. These samples have not been tested using the AFDL YHV1 RT-nPCR due to the difficulties in **Objective 1** when attempting to confirm YHV7 real-time qPCR positives by conventional nPCR. Additional known-YHV1 negative samples will be obtained during FRDC Project 2015/005 to complete the DSp assessment of the tests.

Since developing the AFDL YHV1 TaqMan RT-qPCR, any consignments of commodity prawns testing positive at either screening laboratory using the CSIRO YHV RT-qPCR have been sent to AFDL for confirmation. At AFDL these samples have been retested using the AFDL YHV1 TaqMan RT-qPCR, CSIRO YHV RT-qPCR and OIE YHV/GAV Protocol 2 YHV1 nPCR. Five submissions of prawns from various countries, totalling 15 extracted samples, have been tested to date. Samples from only 1 submission have been confirmed to contain YHV1. Interestingly, this submission was negative by the OIE YHV/GAV Protocol 2 YHV1 nPCR test. However, both real-time TaqMan PCR tests were positive. Additional testing using the AFDL YHV1 RT-nPCR generated an amplicon with a sequence consistent with YHV1. Surprisingly, further examination of the primer binding sites for the OIE YHV/GAV Protocol 2 RT-PCR and YHV1 nPCR showed no mismatches.

Based on testing undertaken thus far, the results indicate that the AFDL YHV1 TaqMan RT-qPCR and YHV1 RT-nPCR are highly sensitive and show good specificity for YHV1 using clinical material. To complete the validation of both tests additional known-positive and known-negative material needs to be obtained and tested. At the completion of the additional testing the information will be submitted for publication and included in the OIE yellow head disease chapter. A recommendation will be made that positive real-time qPCR results be confirmed using conventional RT-PCR and sequencing of amplicons.

Design and validation of YHV7 specific PCR tests

To determine the presence of the recently discovered YHV7 genotype in *P. monodon* inhabiting the study regions in northern Australia, YHV7 TaqMan real-time RT-qPCR and conventional YHV7 RT-nPCR tests were designed at QBP based on an alignment of ORF1b gene sequences available at the time covering the OIE YHV Protocol 1/2 region for genotypes YHV1 to YHV7 and YHV9 (GenBank Acc. KF278563). The tests were transferred to AFDL for evaluation and preliminary validation. When evaluated using the YHV genotype panel (**Table 7**), the ASp of both PCR tests was 100% for YHV7. The ASp of both PCR tests was further confirmed by them not detecting nucleic acids to a selection of exotic (WSSV, IMNV, TSV) and enzootic (IHHNV, MoV, MBV, HPV and SMV) viruses known to infect *P. monodon*. The ASe of both assays was assessed with YHV7 plasmid DNA (provided by QBP). The QBP YHV7 TaqMan RT-qPCR and QBP YHV7 RT-nPCR both detected YHV7 plasmid DNA diluted in water down to a limit of detection as low as 2 to 20 DNA copies per reaction (**Table 10**).

Table 10. QBP YHV7 TaqMan RT-qPCR and QBP YHV7 RT-nPCR tests detection of YHV7 plasmid DNA diluted in water

Plasmid DNA (copies/reaction)	QBP YHV7 TaqMan RT-qPCR (Cт Mean)	QBP YHV7 RT-nPCR
200,000,000	10.2	Positive
20,000,000	13.4	Positive
2,000,000	17.2	Positive
200,000	20.8	Positive
20,000	23.9	Positive
2,000	26.9	Positive
200	30.3	Positive
20	34.2	Positive
2	35.7/Undet	Negative

The DSe and DSp of both the QBP YHV7 TaqMan RT-qPCR and QBP YHV7 RT-nPCR tests have yet to be determined. The primary reason for this is limited known-positive material. FRDC Project 2015/005 will investigate the pathogenicity of YHV7 for *P. monodon* and P. merguiensis, and if pathogenic, material generated in the challenge trials will be used for further validation of the tests.

Design of conventional RT-nPCR tests to detect all known yellow-head-complex virus genotypes

As described above, the OIE YHV Protocol 3 RT-nPCR is limited in identifying YHV genotypes due to the relatively short length of the nPCR amplicon (<100 bp) for sequence analysis and it not reliably detecting genotypes YHV8 and YHV10. As an alternative to this test, a consensus region 1/2 RT-nPCR and the YH30m/YH31m RT-nPCR (**Figure 4B and 4C**) developed at CSIRO-QBP are being used at AFDL. These tests amplify ORF1b gene regions spanning the OIE YHV Protocol 1 and Protocol 2 PCR tests and the OIE YHV Protocol 3 PCR test, and their use enabled the discovery of YHV8 and YHV10. When tested using the YHV genotype panel (which excluded YHV9), both the consensus region 1/2 RT-nPCR and the YH30m/YH31m RT-nPCR were positive for all nucleic acid samples from YHV genotypes YHV1 to YHV10, thus confirming their broad specificity for detecting currently known YHV genotypes (**Table 7**). These results are encouraging, but further validation of the tests is needed before they can be recommended for use in other diagnostic laboratories.

Objective 3: Acquire and/or generate appropriate control nucleic acids specific to the various YHV genotypes for use in YHV-1 or other genotype-specific PCR tests so that their diagnostic specificity can be validated at key diagnostic laboratories (e.g. CSIRO-AAHL), and so they can be made available to state and international laboratories with needs for equivalent diagnostic capabilities

Objective 3 was conducted at AFDL using PCR tests described in **Appendix 2**.

The TaqMan real-time qPCR tests and conventional nPCR tests developed in **Objective 2** for the specific detection of YHV1 or YHV7 will be distributed, on request, to state veterinary diagnostic laboratories. Positive controls will also be provided for these tests, however until additional validation can be undertaken confirmation by conventional PCR and sequence analysis needs to be completed as well. At the time this FRDC-TRF project was being developed, FRDC Project 2014-002 was being considered for approval. One of the objectives of this project was to generate synthetic DNA and RNA positive controls for use with PCR tests for a range of important pathogens of aquatic species. The positive controls designed for the YHV1-specific PCR tests fulfilled two purposes. Firstly, they met **Objectives 2 and 3** of this project, and secondly, they provided confidence in the positive control design approaches to be used for the aquatic pathogen assays identified in FRDC 2014-002.

Design of TaqMan real-time RT-qPCR positive controls

For a TaqMan RT-qPCR test designed to detect an RNA virus, synthetic RNA spanning the region amplified by the test primers is an ideal positive control. However, by including a short unrelated sequence within the synthetic RNA control in combination with employing a second TaqMan probe in the test to detect this sequence, amplification of the positive control RNA can simply and unambiguously be distinguished from amplification of the target viral RNA. By standardising this unrelated sequence and complimentary TaqMan probe in synthetic RNA controls for tests for any pathogen, reagent costs can be minimized and TaqMan RT-qPCR test conditions can be standardized.

For the synthetic RNA controls planned to be used with the TaqMan RT-qPCR tests specific for YHV1 and YHV7, an unrelated sequence complimentary to the TaqMan probe; 5’- VIC-ACCGTCTAGCATCCAGT-TAMRA-3’, described by Snow *et al.* (2009) was inserted either 5’ (YHV7) or 3’ (YHV1) of the virus-specific probe sequence to accommodate its positioning relative to the PCR primers. The synthetic RNAs were also designed so that their 5’- and 3’-termini extended 9 nt beyond the sequences targeted by the forward and reverse primers. As the controls were provided as plasmid DNA constructs, these extensions were included to ensure that the fidelity of the primer binding sites could not be compromised in the process of transcribing RNA synthetically from the plasmid DNA.

Design of conventional RT-nPCR positive controls

For conventional RT-PCR tests, the design approach for the synthetic positive-control RNAs was similar to that used for the TaqMan real-time RT-qPCR tests. However, for these controls, a central 72 nt region of the target sequence was replaced by an unrelated 72 nt sequence. Having the control and virus-specific amplicons identical in size was considered useful for correlating virus-specific amplicon and positive control amplicon sizes when resolved by agarose gel electrophoresis, with amplicon sequence analysis then undertaken to distinguish one from the other. As for the TaqMan

real-time RT-qPCR test controls, the conventional RT-PCR test control for the YHV1 RT-nPCR test was provided as a plasmid DNA construct from which a synthetic RNA could be transcribed as required.

Production and evaluation of synthetic RNA positive controls

Positive controls have been designed for the AFDL YHV1 TaqMan RT-qPCR and YHV1 RT-nPCR tests and the QBP YHV7-TaqMan RT-qPCR and YHV7 RT-nPCR tests, but due to higher priority to assess their performance, only those for use with the YHV1 tests have currently been obtained. As detailed in **Objective 2**, the limit of detection of the positive control synthetic RNA was as expected for the AFDL YHV1 TaqMan RT-qPCR. However, as difficulties were experienced in obtaining known YHV1-positive samples with which to validate the specificity of the tests, a decision to design and obtain the synthetic YHV1-specific positive controls was delayed until further validation can be undertaken. Due to this delay, the performance of the synthetic positive controls for the YHV1 and YHV7 PCR tests will be validated together with comparable positive controls for the other aquatic pathogens as part of the FRDC 2015/005 project. However, to avoid delay in transferring the new AFDL YHV1 PCR test procedures to other diagnostic laboratories, YHV1 positive clinical material is available and can be provided as an interim test positive control. However, as stated above, performance of the assay will need to be confirmed by conventional PCR and sequencing.

Objective 4: Determine the existence and prevalence of other enzootic viruses [e.g. Mourilyan virus (MoV), Monodon baculovirus (MBV), Hepatopancreatic parvovirus (HPV), Spawner-isolated mortality virus (SMV) and Infectious hypodermal and haematopoietic necrosis virus (IHHNV)] in wild *P. monodon* populations in the NT and QLD. In addition, test samples for the exotic viruses WSSV and IMNV given some broodstock are sourced from waters with a higher than usual likelihood of incursion from these pathogens.

Objective 4 was conducted at the QBP using PCR tests described in **Appendix 1**.

PCR Test selection

Details of what PCR tests were selected to detect viruses in addition to GAV/YHV are summarized in **Table 2** and **Appendix 1**. The tests were selected based on them being (i) endorsed by the OIE where appropriate, (ii) published and used widely, (iii) highly sensitive and (iv) able to detect genotypic variants of a virus where these have been reported to exist. The general rationale was thus;

a. To use sensitive and specific TaqMan real-time qPCR tests where available to detect viruses in which the genome region targeted by the PCR primers/probe has not been identified to vary in sequence sufficiently to affect test performance (i.e. MoV, WSSV and IMNV).

b. To use sensitive nPCR tests where validated TaqMan real-time qPCR tests have not been reported for a particular virus (i.e. SMV).

c. To use PCR tests for IHHNV that discriminate infectious virus forms from virus-like homologues in the *P. monodon* genome.

d. To use sensitive nPCR and semi-nPCR tests designed and validated in-house where these provide the most current means of detecting viruses for which many genotypic variants have been identified to exist in prawns from different geographic locations, and where use of tests reported in the literature would not guarantee detection of reported or unknown genetic variants (i.e. MBV and HPV).

Sample selection from the 3 regions in northern Australia for PCR testing

Selected sets of the same gill/pleopod RNA samples used to prepare random-primed cDNA to detect GAV/YHV were examined using the PCR tests for MoV and IMNV (**Table 1**). For the DNA viruses WSSV and IHHNV, DNA was extracted from gill/pleopod tissue of prawns. In most cases, DNA was extracted from tissues of the same prawns used to extract RNA to test for RNA viruses. However, other prawn batches were used in some cases either where tissue was unavailable or tissue amounts were insufficient. For the DNA viruses HPV, MBV and SMV that replicate preferentially in digestive tract tissues, DNA was extracted from hepatopancreas (HP) tissue in cases where this tissue was available (**Table 1**). In cases when HP tissue was not available, DNA extracted from gill/pleopod tissue was tested.

Decapod 18S rDNA

The OIE PCR test for amplifying 18S rDNA from decapods (Lo *et al.* 1996) was used to confirm the integrity/quality of the DNA samples prior to PCR testing for the DNA viruses IHHNV, SMV, MBV, HPV

and WSSV. For the gill or pleopod tissue samples, DNA from 16 prawns selected at random from each of 2 JBG batches, 3 NQ batches and the GoC cohort was tested. The DNA was diluted 1:10 prior to PCR to dilute any PCR inhibitors that might be present. All DNA samples generated the expected 848 bp PCR amplicon (data not shown). For DNA samples extracted from HP tissue, all were diluted 1:5 (due to lower DNA yields relative to the gill/pleopod samples) prior to testing. While all 60 HP DNA samples prepared for the Etty Bay prawns generated the 848 bp PCR amplicon but only 7/40 HP DNA samples from JBG and none of the 51 HP samples from Bramston Beach or 13 HP samples from GoC generated amplicons. Only HP samples which generated the decapod 18S rDNA amplicon were analysed using the various viral PCR tests.

Results for Enzootic viruses

MoV

As summarized in **Table 11**, MoV was detected in 65/153 (43%) of gill/pleopod tissue samples of the prawns originating from NQ locations, only 2/67 (3%) of prawns from GoC locations, and only 4/150 (3%) prawns from JBG. Tissues of all 58 *P. monodon* from the most southerly NQ location of Etty Bay were MoV-positive. Except for 1/58 prawns from Etty Bay and 1/44 prawns from Yorkeys Knob-Taylors Point in which MoV was detected at levels consistent with a high infection load (i.e. Ct value ≤17), all other positives were consistent with MoV existing at very low to moderate infection loads (**Table 11**).

Table 11. MoV TaqMan RT-qPCR data including estimations of infection severity and prevalence in prawns collected from the different regions

		NQ			GoC					JBG
Pathogen level	**C_T value**	**Bramston Beach**	**Etty Bay**	**Yorkeys Knob - Taylor Point**	**Groote Island**	**Vanderlin Islands**	**Mornington Island**	**Weipa**	**Karumba**	
		gill	**gill**	**pleopod**	**Gill (13) & pleopod (54)**					**pleopod**
Very High	≤ 17		1	1						
High	17 <23									
Moderate	23 <29		2							
Low	29 <35		45	3						
Very Low	≥ 35		10	3	1		1			4
UD	UD	51	0	37	7	29	3	20	6	146
Sample No.		51	58	44	8	29	4	20	6	150
Total +ve		65/153 (43%)			2/67 (3%)					4/150 (3%)

UD = undetermined

IHHNV

Using the OIE IHHNV 309F/R PCR test designed to detect infectious IHHNV, the expected 309 bp DNA product was amplified from prawns originating from each of the 3 study regions in northern Australia (**Table 12**). The PCR test was positive for between 3% and 10% of the prawns tested from NQ locations (7% overall), for 24% and 17% of the GoC prawns tested from Weipa and Karumba, respectively (10% overall), and for 3% of the 136 prawns tested from JBG. Whether or not the

somewhat higher percentages of PCR-positives obtained for the *P. monodon* sampled from Weipa and Karumba are representative of prawns inhabiting these regions cannot be predicted with any accuracy due to the relatively low numbers of prawns available for testing.

SMV

SMV was detected in only 2/151 (1%) prawns collected from NQ locations, in none of the 50 prawns tested from GoC locations and in only 2/136 (2%) JBG prawns from QLD Hatchery 2 for which pleopod DNA was tested (**Table 12**). Using DNA extracted from HP tissue, which was not available for most prawn batches, SMV was detected in 1/60 (2%) prawns from Etty Bay in NQ and 1/7 (14%) JBG prawns. Based on these data, it is impossible to predict whether the availability of HP or other digestive tract tissue sources (e.g. stomach, faeces) would have resulted in higher SMV prevalence estimates.

MBV

MBV was detected in DNA extracted from gill/pleopod of 2/151 (1%) prawns from NQ locations and in 1/50 (2%) prawns from GoC locations (**Table 12**). MBV was not detected among the batch of JBG prawns from QLD Hatchery 2 that were tested. MBV was also not detected in any of the 60 Etty Bay prawns irrespective of whether DNA was extracted from HP rather than gill tissue. The prevalence data on MBV was likely compromised by the unavailability of HP tissue to test.

HPV

HPV was detected in gill/pleopod DNA samples from prawns sampled from the 3 NQ locations (3-28%, 11% overall), from 4 of the 5 GoC locations (7-25%, 14% overall) and from the JBG prawns examined from QLD Hatchery 2 (5%) (**Table 12**). Testing of DNA extracted from HP tissue from the Etty Bay group of prawns identified 24 (40%) PCR-positives compared to only 2 (3%) when gill DNA was tested. These data indicated that the use of gill/pleopod DNA resulted in the HPV prevalence being underestimated considerably.

Results for Exotic viruses

IMNV

IMNV was not detected in any samples. The gill/pleopod sample set included 153 prawns from NQ locations, 67 prawns from GoC locations and 150 prawns from JBG (total *P. monodon* tested= 372).

WSSV

WSSV was not detected in any samples. The gill/pleopod sample set included 151 prawns from NQ locations, 50 prawns from GoC locations and 136 JBG prawns (total *P. monodon* tested= 337).

Table 12. Summary of PCR test data obtained for the enzootic DNA viruses IHHNV, SMV, MBV and HPV

Region and Location	Sample No. and types tested			IHHNV	SMV		MBV		HPV	
				No. +ve	No. +ve		No. +ve		No. +ve	
	Gill	Pleo	HP	Gill + Pleo	Gill + Pleo	HP	Gill + Pleo	HP	Gill + Pleo	HP
JBG										
QLD Hatchery 1			7			1 (14%)		0		0
QLD Hatchery 2		136		4 (3%)	2 (2%)		0		7 (5%)	
GoC										
Groote Island	5	3		0	0		0		1	
Vanderlin Islands	7	8		0	0		0		1	
Mornington Island	1	3		0	0		0		1	
Weipa	0	17		4 (24%)	0		1		4	
Karumba	0	6		1 (17%)	0		0		0	
GoC Total	13	37		5 (10%)	0		1 (2%)		7 (14%)	
NQ										
Bramston Beach	51			5 (10%)	1 (2%)		2 (4%)		3 (6%)	
Etty Bay	60		60	4 (7%)	0	1 (2%)	0	0	2 (3%)	24 (40%)
Yorkeys Knob-Taylor Point	40			1 (3%)	0		0		11 (28%)	
NQ Total	151		60	10 (7%)	1 (<1%)	1 (2%)	2 (1%)	0	16 (11%)	24 (40%)

Pleo = pleopod, HP = hepatopancreas

Conclusions

Objective 1: Determine what GAV/YHV genotypes exist and their relative prevalence in wild *P. monodon* populations in NT/WA/QLD from which broodstock are captured for aquaculture purposes.

GAV was detected among *P. monodon* trawled from each of the 3 QLD East Coast locations, consistent with results of analysis of broodstock samples from hatcheries over a decade ago (Cowley *et al.* 2000, Walker *et al.* 2001). YHV6, which of all known genotypes is most closely related to GAV was also detected in samples from Etty Bay. It is not unexpected that it has not been reported previously due to its closeness in sequence to GAV allowing it to be amplified by the conventional and TaqMan GAV qPCR tests. GAV was also detected among prawns originating from the GoC and JBG.

YHV7 was detected in samples from all of the regions. However, the presence of YHV7 was only confirmed by sequence analysis of samples originating from JBG and F1 progeny from a QLD farm. The detection of YHV7, by TaqMan RT-qPCR, in stored nucleic acid from prawns from a QLD farm in 2008 suggests that this genotype was already present in QLD.

Objective 2: Revise PCR test designs as necessary to ensure their specificity, particularly in discriminating the highly virulent YHV1 strain that emerged in Thailand in the early 1990s from GAV and the other known YHV genotypic variants that appear to be far less pathogenic, and make these tests available for publication in the OIE Diagnostic Manual chapter for yellow head disease.

As a lack of specificity was identified in a number of the current OIE assays recommended for the detection of YHV1, new YHV1-specific conventional and TaqMan real-time qPCR tests were developed and evaluated. Testing indicated that the new assays are sensitive and specific only for YHV1. Due to a lack of known YHV1-positive material, validation has been delayed and it is anticipated that if material cannot be sourced from overseas it will be generated during FRDC Project 2015/005 investigating the infectivity of YHV7, YHV8 and YHV10 to *P. monodon* and *P. merguiensis*. Prawns will be infected with YHV1 as a positive infection control during these trials.

To help meet a component of **Objective 1**, new YHV7-specific conventional and TaqMan RT-qPCR tests were developed and evaluated. The YHV7 assays proved to be highly specific for YHV7, and will be valuable tools for state veterinary laboratories or other regional laboratories. Test validation is not yet complete due to limited known-positive material, therefore any YHV7 TaqMan RT-qPCR positive test results will need to be confirmed by conventional PCR and sequence analysis.

A lack of specificity was also identified in a YHV1-specific real-time assay (Tang-Nelson and Lightner, 2006) and a GAV-specific real-time assay (de la Vega et al, 2004). Either these tests need to be revised or positive results should be confirmed by conventional PCR and sequence analysis. Similarly, results obtained using the CSIRO YHV TaqMan RT-qPCR must be confirmed by conventional PCR and sequence analysis.

Objective 3: Acquire and/or generate appropriate control nucleic acids specific to the various YHV genotypes for use in YHV-1 or other genotype-specific PCR tests so that their diagnostic specificity can be validated at key diagnostic laboratories (e.g. CSIRO-AAHL), and so they can be made available to state and international laboratories with needs for equivalent diagnostic capabilities.

Due to inadequate validation of the new YHV1 and YHV7 assays, synthetic RNA positive control material was not developed as the assays may require changes. Development of positive controls will be undertaken as a component of FRDC 2014-002 “Aquatic Animal Health Subprogram: Development of stable positive control material and development of internal controls for molecular tests for detection of important endemic and exotic pathogens.” In the interim, positive control material generated from animals infected *in vivo* can be provided. However, any laboratories using the new YHV1 and YHV7 assays would do so in collaboration with AFDL in order to generate and collate additional validation data.

Objective 4: Determine the existence and prevalence of other enzootic viruses [e.g. Mourilyan virus (MoV), Monodon baculovirus (MBV), Hepatopancreatic parvovirus (HPV), Spawner-isolated mortality virus (SMV) and Infectious hypodermal and haematopoietic necrosis virus (IHHNV)] in wild *P. monodon* populations in the NT and QLD. In addition, test samples for the exotic viruses WSSV and IMNV given some broodstock are sourced from waters with a higher than usual likelihood of incursion from these pathogens.

Enzootic viruses (MoV, IHHNV, SMV, MBV and HPV)

With regard to MBV, IHHNV and SMV, PCR analyses identified no evidence of the pathogen at many locations but when detected the prevalence was generally low. In contrast, MoV and HPV were detected in P. monodon collected from most locations. Due to the timing and options available (mostly non-lethal) for collecting samples from P. monodon inhabiting different regions in northern Australia, collections were weighted to pleopod and/or gill tissue. Therefore, the accuracy of the prevalence data at most locations, for MBV, SMV and HPV in particular, had the potential to be underestimated due to the lack of HP tissue.

Exotic viruses (IMNV and WSSV)

IMNV and WSSV were not detected in any of the *P. monodon* tested from any location. The results provide additional evidence for Australia’s claims for freedom from these pathogens.

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Implications

Detection of YHV7 in *P. monodon* broodstock sampled from QLD hatcheries soon after their arrival from JBG implies that this genotype is entering QLD via translocation of prawns from this region. Although unable to be confirmed by conventional PCR and sequencing, YHV7 was detected by TaqMan RT-qPCR in *P. monodon* from NQ locations commonly trawled to supply broodstock to hatcheries and archived NQ farmed *P. monodon* nucleic acids. Additional targeted surveillance in QLD is required to clarify the distribution of YHV7.

The YHV1- and YHV7-specific PCR tests developed will be a useful resource for State Government veterinary diagnostic services laboratories with regulatory responsibilities including prawn aquaculture disease investigations. When validated, the YHV1 tests will also be a useful resource for commercial diagnostic laboratories undertaking testing for YHV1 import certification requirements. The lack of specificity of a number of assays highlights the need for adequate and ongoing validation of assays. This also highlights the need to use multiple assays for significant diseases and to confirm results by conventional PCR and sequence analysis.

PCR testing identified no evidence of the exotic viruses YHV1, WSSV and IMNV occurring in the *P. monodon* tested from any location.

Recommendations

To more fully assess what risks to prawn aquaculture are posed by the YHV7 genotype *P. monodon*, its pathogenicity in farmed *P. monodon* and other species of fisheries and aquaculture importance needs to be determined in experimental challenge trials. This is now being undertaken as a component of FRDC 2015-005 "Aquatic Animal Health Subprogram: Determining the susceptibility of Australian *Penaeus monodon* and *P. merguiensis* to newly identified enzootic (YHV7) and exotic (YHV8 and YHV10) Yellow head virus (YHV) genotypes". These infectivity trials will also generate material required for validation of the newly designed YHV1 and YHV7-specific assays. Distribution of the assays, when more adequately validated, to state diagnostic laboratories should be undertaken in conjunction with proficiency testing to verify the successful adoption of the new tests.

Further development

Synthetic RNA positive control material was not produced as planned during the project. However, after additional validation has been undertaken for the new YHV1 and YHV7 PCR tests and the assays have been determined to be fit for purpose, these controls will be developed, evaluated and distributed as part of FRDC Project 2014-002 "Aquatic Animal Health Subprogram: Development of stable positive control material and development of internal controls for molecular tests for detection of important endemic and exotic pathogens".

Extension and Adoption

Information concerning this project has and will continue to be provided to industry, researchers and government regulators. To date, the following presentations have been made or are planned:

Cowley JA (2014) Virus prevalence in Black Tiger prawns inhabiting Northern Australia and revised PCR tests for YHV. Oral presentation, 2014 Ridley Aqua Feed Australian Prawn and Barramundi Farmers Symposium, Sea World Resort, Gold Coast, 6-7 August 2014.

Cowley JA (2015) Viral Prevalence project update. Oral presentation, 2015 Ridley Australian Prawn & Barramundi Farmers Symposium, Sea World Resort, Gold Coast, 31 July 2015.

Moody NJG, Mohr PG, Hoad J, Williams LM, Bowater RO, Cummins DM, Cowley JA and Crane MStJ (2015) The yellow head disease complex: it's complex. Oral presentation, Third Australasian Scientific Conference on Aquatic Animal Health, The Pullman Reef Hotel, Cairns, 6-10 July 2015.

Moody NJG (2015) The Yellow Head Disease Complex: detection and identification of new genotypes. Oral presentation, 2015 Ridley Australian Prawn & Barramundi Farmers Symposium, Sea World Resort, Gold Coast, 31 July 2015.

In addition, a copy of the approved final report will be forwarded to all stakeholders.

Project materials developed

Talking Points were developed regarding the detection of YHV7, YHV8 and YHV10 for distribution to state governments and industry. These are included as Appendix 4. These were sent to Tony Charles, representing the APFA with the full application for FRDC 2015-005 “Aquatic Animal Health Subprogram: Determining the susceptibility of Australian *Penaeus monodon* and *P. merguiensis* to newly identified enzootic (YHV7) and exotic (YHV8 and YHV10) Yellow head virus (YHV) genotypes” by email on 2 April, 2015.

A manuscript describing the detection of YHV7 has been published:

Mohr PG, Moody NJG, Hoad J, Williams LM, Bowater RO, Cummins DM, Cowley JA and Crane MStJ (2015) Discovery of a new genotype of yellow head virus (YHV7) in giant tiger shrimp (*Penaeus* monodon) indigenous to northern Australia. Diseases of Aquatic Organisms 115, 263-268.

Appendices

Appendix 1: List of researchers and project staff

CSIRO-QBP

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Appendix 2 PCR test primers and thermal cycling conditions as used at QBP

PCR Test	Primer/ Probe	Sequence (5'-3')	Thermal Cycling Conditions	Amplicon	Reference
Real-time (TaqMan) PCR					
GAV qPCR	GAV-QPF1 GAV-QPR1 GAV-Pr1 (FAM-TARMA)	GGGATCCTAACATCGTCAACGT AGTAGTATGGATTACCCTGGTGCAT TCAGCCGCTTCCGCTTCCAATG	95°C 2 min, 95°C 15 sec - 60°C 30 sec (40 cycles)	81 bp	De la Vega *et al.* (2004)
QBP YHV7 qPCR	qYHV7-F1 qYHV7-R1 qYHV7-Pr1 (FAM-TAMRA)	CATCCAACCTATCGCCTACA TGTGAAGTCCATGTGAACGA CAACGACAGACACCTCATCCGTGA	As per GAV qPCR	79 bp	QBP unpublished
MoV qPCR	MoV-QPF1 MoV-QPR1 MoV-Pr1 (FAM-MGB)	TGTTACAAGCACACTGCATCTCA GCTAGGGCAGACCACTTCACA CAATCCATGATTGACATGAA	As per GAV qPCR	67 bp	Rajendran *et al.* (2006)
WSSV qPCR	WSSV-QPF1 WSSV-QPR1 WSSV-Pr1 (FAM-TAMRA)	CCGACGCCAAGGGAACT TTCAGATTCGTTACCGTTTCCA CGCTTCAGCCATGCCAGCCG	As per GAV qPCR	80 bp	East *et al.* (2004)
IMNV qPCR	IMNV412F IMNV545R IMNV probe (FAM-TAMRA)	GGGATCCTAACATCGTCAACGT AGTAGTATGGATTACCCTGGTGCAT TCAGCCGCTTCCGCTTCCAATG	As per GAV qPCR	134 bp	Andrade *et al.* (2007)
Conventional PCR					
CSIRO YHV7 PCR	YHV7-F1a YHV7-R1a	CCTACACGCATGCTCTCTCTATG GGTGTCTGTCGTTGTGTATAGCT	94°C 2 min, 94°C 30 sec - 58°C 30 sec - 72°C 45sec (35 cycles) 72°C 7 min	788 bp	QBP unpublished
CSIRO YHV7 nPCR	YHV7-F2a YHV7-R2a	CAAACACCAACCGACATTCAGT GCGACAGTGCTTGAAGACTTTAG	94°C 2 min, 94°C 30 sec - 58°C 30 sec - 72°C 30 sec (35 cycles) 72°C 7 min	412 bp	QBP unpublished
YH30m PCR	YH30-F1m YH30-R1m	TACCAYTCAAACATCATYAAYAAYCAYCA GAGATGATYTGRTKCTTRAAYTTCTGRAA	94°C 2 min, 94°C 30 sec - 55C 30 sec - 72°C 50sec (35 cycles) 72°C 7 min	1001 bp	Modified from Wijegoonawardane *et al.* 2008a
YH31m nPCR	YH31-F2m YH31-R2m	CTCARATCCATGCMATYTGGGARTCHTC AGTTTGGCRCGRATRTTRGTRAGRAT	94°C 2 min, 94°C 30 sec - 55C 30 sec - 72°C 40sec (35 cycles) 72°C 7 min	721 bp	Modified from Wijegoonawardane *et al.* 2008a

GAV PCR	GAV 5 GAV 6	AACTTTGCCATCCTCGTCAC TGGATGTTGTGTGTTCTCAAC	94°C 2 min, 94°C 30 sec - 58°C 30 sec - 72°C 40sec (35 cycles) 72°C 7 min	618 bp	Cowley *et al.* (2000)
GAV nPCR	GAV 1 GAV 2	ATCCATACTACTCTAAACTTCC GAATTTCTCGAACAACAGACG	94°C 2 min, 94°C 30 sec - 58°C 30 sec - 72°C 30sec (35 cycles) 72°C 7 min	317 bp	Cowley *et al.* (2000)
HPV PCR	HPV-409F HPV-661R	ATGAAYCAYYTDCAGGCATTYGARATG CCTCKYTGTGTRTGRRCRTAIGGCAT	94°C 2 min, 94°C 30 sec - 52°C 30 sec - 72°C 40sec (35 cycles) 72°C 7 min	756 bp	Salote Waqairatu QPB unpublished
HPV semi-nPCR	HPV-552F HPV-661R	AGGAARGARATHCARTCRGGYATGGA As above	94°C 2 min, 94°C 30 sec - 50°C 30 sec - 72°C 30sec (35 cycles) 72°C 7 min	327 bp	Salote Waqairatu QBP unpublished
MBV PCR	MBV-F1(1.4F) MBV-R1(1.4R)	CGATTCCATATCRGCCGAATA TTGGCATGCACTCCCTGAGAT	As per HPV RT-PCR	533 bp	Salote Waqairatu QBP unpublished
MBV nPCR	MBV-NF2 MBV-NR2	TATATYCCAATCGYGTCTGCGAT CGCTWATGGGRCACAAGTCTC	94°C 2 min, 94°C 30 sec - 54°C 30 sec - 72°C 30sec (35 cycles) 72°C 7 min	361 bp	Salote Waqairatu QBP unpublished
SMV PCR	SMV-200F SMV-200R	TAGCTATTTTTTGGTCGTCTG GCAATTTACCAGTGTTTGAAG	94°C 2 min, 94°C 30 sec - 58°C 30 sec - 72°C 30sec (35 cycles) 72°C 7 min	207 bp	Owens *et al.* (2003)
SMV nPCR	SMV-22798F SMV-22798R	CACTTCTTTTTTTGAAGTTCA GAAAATAGGAAACAAGTGGC	94°C 2 min, 94°C 30 sec - 53°C 30 sec - 72°C 30sec (35 cycles) 72°C 7 min	147 bp	Owens *et al.* (2003)
IHHNV PCR	IHHNV-392F IHHNV-392R	GGGCGAACCAGAATCACTTA ATCCGGAGGAATCTGATGTG	94°C 2 min, 94°C 30 sec - 55°C 30 sec - 72°C 40sec (35 cycles) 72°C 7 min	309 bp	OIE test to detect infectious virus types
Decapod 18S rDNA PCR	20s1 - 143F 20a1 - 145R	TGCCTTATCAGCTNTCGATTGTAG TTCAGNTTTGCAACCATACTTCCC	94°C 2 min, 94°C 30 sec - 55°C 30 sec - 72°C 50sec (35 cycles) 72°C 7 min	848 bp	Lo *et al.* (1996)

Appendix 3. PCR test primers and thermal cycling conditions as used at AFDL

PCR Test	Primer/ Probe	Sequence (5'-3')	Thermal Cycling Conditions	Amplicon	Reference
Real-time (TaqMan) PCR					
AFDL YHV1 RT-qPCR	YHV1-12-qF YHV1-12-qR YHV1-12-qPr (FAM-TAMRA)	AGTCTACAGTGCTCTGATCT GATTCTTGAAGCGCATGAGT TCTCATGTGTCATGATATTCTCAAGCGAGT	48°C 30 min, 95°C 10 min, 95°C 10 sec - 60°C 60 sec (45 cycles)	150 bp	AFDL unpublished
QBP YHV7 RT-qPCR	qYHV7-F1 qYHV7-R1 qYHV7-Pr1 (FAM-TAMRA)	CATCCAACCTATCGCCTACA TGTGAAGTCCATGTGAACGA CAACGACAGACACCTCATCCGTGA	As per AFDL YHV1 RT-qPCR	79 bp	QBP unpublished
CSIRO YHV RT-qPCR	YHV-QPF1 YHV-QPR1 YHV probe (FAM-TAMRA)	CAAAGGATCCAAACATTGTGAATGT ATGGATTGCCCTGGTGCAT TCAGTCGTTTCCGCTTCCAGTGTATCTG	As per AFDL YHV1 RT-qPCR	78 bp	QBP unpublished
Tang YHV RT-qPCR	YHV141F YHV206R TangPr (FAM-TAMRA)	CGTCCCGGCAATTGTGATC CCAGTGACGTTCGATGCAATA CCATCAAAGCTCTCAACGCCGTCA	As per AFDL YHV1 RT-qPCR	66 bp	Tang-Nelson and Lightner (2001), Aranguren *et al.* (2012)
de la Vega GAV RT-qPCR	GAVQPF1 GAVQPR1 GAVprobe1 (FAM-TAMRA)	GGGATCCTAACATCGTCAACGT AGTAGTATGGATTACCCTGGTGCAT TCAGCCGCTTCCGCTTCCAATG	As per AFDL YHV1 RT-qPCR	81 bp	de la Vega *et al.* (2004)
Conventional PCR					
OIE YHV Protocol 1 RT-PCR	10F 144R	CCGCTAATTTCAAAAACTACG AAGGTGTTATGTCGAGGAAGT	50°C 30 min, 94°C 2 min, 94°C 30 sec - 58°C 45 sec - 68°C 45 sec (40 cycles) 68°C 7 min	135 bp	Wongteerasupaya *et al.* (1997)
OIE YHV/GAV Protocol 2 RT-PCR	GY1 GY4	GACATCACTCCAGACAACATCTG GTGAAGTCCATGTGTGTGAGACG	50°C 30 min, 95°C 2 min, 95°C 30 sec - 66°C 30 sec - 68°C 45 sec (35 cycles) 68°C 7 min	794 bp	Cowley *et al.* (2004)
OIE YHV/GAV Protocol 2 – YHV1 nPCR	GY2 Y3	CATCTGTCCAGAAGGCGTCTATGA ACGCTCTGTGACAAGCATGAAGTT	95°C 15 min, 95°C 30 sec - 66°C 30 sec - 72°C 45 sec (35 cycles) 72°C 7 min	406 bp	Cowley *et al.* (2004)
OIE YHV/GAV Protocol 2 – GAV nPCR	GY2 G6	As above GTAGTAGAGACGAGTGACACCTAT	As per OIE YHV/GAV Protocol 2 YHV1 nPCR	277 bp	Cowley *et al.* (2004)

OIE YHV Protocol 3 RT-PCR	YC-F1a YC-F1b YC-R1a YC-R1b	ATCGTCGTCAGCTACCGCAATACTGC ATCGTCGTCAGYTAYCGTAACACCGC TCTTCRCGTGTGAACACYTTCTTRGC TCTGCGTGGGTGAACACCTTCTTGGC	50°C 55 min, 95°C 2 min, 94°C 45 sec - 60°C 45 sec - 68°C 45 sec (35 cycles) 68°C 7 min	359 bp	Wijegoonawardane *et al.* (2008b)
OIE YHV Protocol 3 nPCR	YC-F2a YC-F2b YC-R2a YC-R2b	CGCTTCCAATGTATCTGYATGCACCA CGCTTYCARTGTATCTGCATGCACCA RTCDGTGTACATGTTTGAGAGTTTGTT GTCAGTGTACATATTGGAGAGTTTRTT	95°C 15 min, 95°C 30 sec - 66°C 30 sec - 72°C 45 sec (35 cycles) 72°C 7 min	147 bp	Wijegoonawardane *et al.* (2008b)
AFDL YHV1 RT-PCR	YHV1-3-RTF YHV1-3-RTR	CTTGACATTCAAATCCCCATC CAGATAGGATGCTTGGTGTT	50°C 30 min, 94°C 2 min, 94°C 30 sec - 60°C 30 sec - 68°C 60 sec (35 cycles) 68°C 7 min	561 bp	AFDL unpublished
AFDL YHV1 nPCR	YHV1-3-nF YHV1-3-nR2	CACTGTCCACACATCGCAAG TGCTTAGCTAGATCGGGATTG	95°C 15 min, 94°C 30 sec - 60°C 30 sec - 72°C 30 sec (35 cycles) 72°C 7 min	483 bp	AFDL unpublished
QBP YHV7 RT-PCR	YHV7-F1a YHV7-R1a	CCTACACGCATGCTCTCTCTATG GGTGTCTGTCGTTGTGTATAGCT	50°C 30 min, 94°C 2 min, 94°C 45 sec - 55°C 45 sec - 68°C 60 sec (35 cycles) 68°C 7 min	788 bp	QBP unpublished
QBP YHV7 nPCR	YHV7-F2a YHV7-R2a	CAAACACCAACCGACATTCAGT GCGACAGTGCTTGAAGACTTTAG	95°C 15 min, 94°C 45 sec - 55°C 45 sec - 72°C 60 sec (35 cycles) 72°C 7 min	412 bp	QBP unpublished
QBP Consensus Region 1/2 RT-PCR	GAV210mF GAV211mR	TCGTGCAACATYCTYAAGATGGA TGCGGGTTTCTTRGTGTCRTC	50°C 30 min, 95°C 2 min, 94°C 30 sec - 50°C 30 sec - 68°C 60 sec (35 cycles) 68°C 7 min	1239 bp	Modified primer GAV210F and GAV211R QBP unpublished
Consensus Region 1/2 nPCR	GY1 GY5	As above GAGCTGGAATTCAGTGAGAGAACA	95°C 15 min, 94°C 30 sec - 50°C 30 sec - 72°C 60 sec (40 cycles) 72°C 7 min	1047 bp	Primers described in Cowley *et al.* (2004)
QBP YH30m RT-PCR	YH30-F1m YH30-R1m	TACCAYTCAAACATCATYAAYAAYCAYCA GAGATGATYTGRTKCTTRAAYTTCTGRAA	As per CSIRO YHV7 RT-PCR	1001 bp	Modified from Wijegoonawardane *et al.* 2008a
QBP YH31m nPCR	YH31-F2m YH31-R2m	CTCARATCCATGCMATYTGGGARTCHTC AGTTTGGCRCGRATRTTRGTRAGRAT	As per CSIRO YHV7 nPCR	721 bp	Modified from Wijegoonawardane *et al.* 2008a

Appendix 4: YHV Genotype talking points

Draft talking points – New YHV-complex genotypes detected in imported commodity prawns from China

- In 2013 a new YHV-complex genotype, YHV-7, was detected in diseased *Penaeus monodon* that had been submitted for testing from a Queensland farm. It is unknown what involvement YHV-7 had in the disease, and as yet the pathogenicity of the newly-detected YHV-7 is unknown.
- The only other YHV-complex genotype that occurs in Australia is Gill-associated Virus (GAV, also known as YHV-2). The highly pathogenic YHV-complex genotype 1 (YHV-1) that causes Yellowhead disease is exotic to Australia.
- Consignments of imported raw prawns that are not deemed highly processed are batch-tested for YHV genotype 1 (YHV-1) on arrival in Australia. Each consignment is held at a quarantine- approved premises pending test results.
- In 2014, several consignments of imported prawns originating from China tested positive using an assay thought to be specific for YHV genotype 1. Additional testing was undertaken at the CSIRO Australian Animal Health Laboratory (AAHL), which also hosts the OIE Reference Laboratory for Yellowhead disease. CSIRO AAHL testing indicated that the positive test results were the result of novel Yellowhead virus genotypes present in the prawns. These results indicated a lack of specificity of the screening YHV-1 test being used.
- To date, two new YHV-complex genotypes have been detected in prawns imported from China, designated YHV-8 and YHV-10. A third YHV-complex genotype, designated YHV-9, has been reported by Chinese authorities but has not been detected in commodity prawns imported into Australia.
- The pathogenicity of the newly-detected endemic (YHV-7) and exotic (YHV-8, YHV-9 and YHV-10) YHV-complex genotypes is unknown.
- CSIRO AAHL is re-evaluating the specificity of OIE-recommended tests for YHV genotype 1 and related genotypes. This work is ongoing and AAHL is evaluating new PCR assays on a range of YHV-complex genotypes from different geographic locations. The new YHV assays will be validated by CSIRO AAHL and included in a revised OIE Aquatic Manual chapter for Yellowhead disease as soon as possible.
- In the interim, imported prawns that test positive for YHV, will be re-tested by AAHL to confirm if the positive signal is due to YHV-1.
- In addition, a research project is being developed to determine if Australian *Penaeus monodon* are susceptible to the newly identified enzootic (YHV-7) and exotic (YHV-8 and YHV-10) Yellowhead virus (YHV) genotypes.
- A summary of the new genotypes is provided below.

Genotype 7 – 2012	Australia: detected in broodstock prawns, unpublished.
Genotype 8 – 2014	China: detected by Australian laboratories in imported commodity prawns from China, unpublished.
Genotype 9 – 2012	China: detected by a Chinese laboratory and the sequence published on GenBank.
Genotype 10 – 2014	China: detected by Australian laboratories in imported commodity prawns from China, unpublished.