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TaqMan real-time and conventional nested PCR tests specific to *yellow head virus* genotype 7 (YHV7) identified in giant tiger shrimp in Australia

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ABSTRACT

In 2013, a unique seventh yellow head virus genotype (YHV7) was detected in Black Tiger shrimp (*Penaeus monodon*) broodstock that suffered high mortality following their capture from Joseph Bonaparte Gulf (JBG) in northern Australia. To assist with its diagnosis and assessment of its distribution, prevalence and pathogenicity, YHV7-specific TaqMan real-time qPCR and conventional nested PCR primer sets were designed to ORF1b gene sequences divergent from the other YHV genotypes. Using high ($\geq 10^8$) copies of plasmid (p)DNA controls containing ORF1b gene inserts of representative strains of YHV genotypes 1–7, both PCR tests displayed specificity for YHV7. Amplifications of serial 10-fold dilutions of quantified YHV7 pDNA and synthetic ssRNA showed that both tests could reliably detect 10 genome copies. Pleopods/gills from wild *P. monodon* sourced from locations in geographically disparate regions across northern Australia as well as 96 juveniles (48 either appearing normal or displaying signs of morbidity) from a commercial pond experiencing mortalities were screened to partially validate the diagnostic capacity of the qPCR test. Based on these data and PCR primer/probe sequence mismatches with other newly identified YHV genotypes, both YHV7-specific PCR tests should prove useful in the sensitive detection and discrimination of this genotype from YHV 2 (gill-associated virus) and YHV6 that can occur in Australian *P. monodon*, as well as from YHV genotypes currently listed as exotic to Australia.

1. Introduction

In the early to mid-1990s, yellow head virus (YHV1) and gill-associated virus (GAV; YHV2) emerged as serious pathogens of farmed Giant Tiger shrimp (*Penaeus monodon*) in Thailand and Australia, respectively (Limsuwan, 1991; Boonyaratpalin et al., 1993; Chantanachookin et al., 1993; Spann et al., 1998; Walker et al., 2001; Callinan and Jiang, 2003). Both viruses form rod-shaped enveloped virions (Chantanachookin et al., 1993; Lu et al., 1994; Spann et al., 1995, 1998) decorated with spikes formed by 2 glycoproteins (Cowley and Walker, 2002; Jitrapakdee et al., 2003). As they are also closely related genetically (Cowley et al., 1999; Sittidilokratna et al., 2002) and possess a long (~26.2–26.6 kb) positive-sense ssRNA genome with structural, transcriptional and translational features conserved among viruses classified within the Order *Nidovirales*, GAV was designated type species of the genus *Okavirus*, family *Roniviridae* (Cowley et al., 2000a, 2002a,b, 2012; Cowley and Walker, 2002; Sittidilokratna et al., 2002, 2008; de Groot et al., 2012; Wongteerasupaya et al., 1995).

Among *P. monodon* sampled between 1997 and 2003 from regions distributed widely across its natural Indo-Pacific host range including Eastern Africa, India, Australia and countries in Southeast Asia, PCR amplification and sequence analysis of short regions in the ORF1b gene identified at least 4 other YHV genotypes (YHV3, 4, 5 and 6) (Walker et al., 2001; Soowannayan et al., 2003; Wijegoonawardane et al., 2008a). The discovery of these genotypes lent support to a hypothesis that YHV might exist as a complex of genotypes having evolved in geographically segregated populations of *P. monodon* (Walker et al., 2001), and YHV and GAV were thus assigned as YHV genotypes 1 (YHV1) and 2 (YHV2), respectively (Wijegoonawardane et al., 2008a; Cowley et al., 2012). As yet there is no reported evidence for YHV3, 4, 5, or 6 causing disease in shrimp. However, virulent virus strains consistent in sequence with YHV1 have been detected in Pacific white shrimp (*Penaeus vannamei*) and Pacific blue shrimp (*Penaeus stylirostris*) being farmed along the Mexican Pacific coast in 1999–2000 and subsequently in *P. stylirostris* tested from the Gulf of California (de la Roas-Vélez et al., 2006; Castro-Longoria et al., 2008). YHV1 has also been

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Table 1
Sequences and calculated annealing temperatures of the PCR primers and probes employed in the YHV7 TaqMan real-time qPCR and conventional RT-nested PCR tests.

PCR test	Primers/probe	Sequence (5'-3')	Length (nt)	Tm (°C)		Amplicon length (bp)	Position in YHV1 EU487200.1
				Primer 3	TFS		
YHV7 qPCR	qYHV7-F1	CATCCAACCTATCGCCTACA	20	59.5	55.4	79	13,564-13,585
	qYHV7-F2	ACCTATCGCCTACACAGCTA	20	56.1	56.2	73	13,570-13,589
	qYHV7-R1	TGTGAAGTCCATGTGAACGA	20	59.7	55.0		13,624-13,642
	qYHV7-Pr1	[6FAM]CAACGACAGACACCTCATCCGTGA[BHQ1]	24	69.7	62.6		13,594-13,617
YHV7 PCR	YHV7-F1a	CCTACACGCATGCTCTCTCTATG	23	66.2	58.6	788	12,820-12,842
	YHV7-R1a	GGTGTCTGTCGTTGTGTATAGCT	23	63.4	57.8		13,585-13,607
YHV7 nPCR	YHV7-F2a	CAAACACCAACCGACATTCAGT	22	67.3	57.8	412	13,088-13,108
	YHV7-R2a	GCGACAGTGCTTGAAGACTTTAG	23	65.4	57.7		13,476-13,498
GAV210/211	GAV210m	TCGTGCAACATYCTYAAGATGGA	23	61.9		1239	12,749-12,771
	GAV211m	TGCGGGTTTCTTRGTGTCYTC	21	61.5			13,967-13,987

Primer Tm values based on default settings in Primer 3 v0.4.0, Thermo Fisher Scientific (TFS) Tm Calculator, Tm = melt temp, PCR primers GAV210 m and GAV211m.

associated with disease in *P. vannamei* farmed in Thailand (Senapin et al., 2010).

More recently, a seventh unique YHV genotype (YHV7) was detected during a disease investigation in *P. monodon* originating from Joseph Bonaparte Gulf (JBG) in northern Australia (Mohr et al., 2015) and a virus assigned as YHV genotype 8 has been detected in Chinese white shrimp (*Penaeus chinensis*) farmed in several provinces in China (Liu et al., 2014; Zhu et al., 2016; Dong et al., 2017). In addition to these genotypes, YHV1 has been segregated into sub-genotypes YHV1a/1b based on the presence of a significant deletion in the ORF3 gene sequence of the YHV1b genotype that would result in a 51 amino acid deletion at the gp116 glycoprotein N-terminus (Sittidilokratna et al., 2009). A putative YHV1b/YHV5 recombinant virus has also been described (Gangnonngiw et al., 2009), and viruses with recombinant genomes comprising permutations of ORF1a/1b gene and structural gene regions of strains of YHV2, YHV3 or YHV5 have been detected at high prevalence in *P. monodon* sampled from various countries in Southeast Asia (Wijegoonawardane et al., 2009). Recently, YHV6 has been reported to occur in wild *P. monodon* in Australia (Cowley et al., 2015), YHV3 has been detected in *P. monodon* farmed in China (Chen et al., 2018) and genotypes distinct from YHV1 to 8 have been detected in uncooked commodity shrimp imported into Australia from China (N.J. Moody et al., unpublished data).

To assist in minimizing disease impacts of YHV7 in *P. monodon* farmed in Australia and in determining its prevalence, distribution and pathogenicity, reported here are YHV7-specific TaqMan real-time qPCR and conventional nested PCR tests.

2. Materials and methods

2.1. Shrimp samples

Pleopod or gill tissue was sampled from 815 *P. monodon* collected between 2013 and 2019 from various locations spread widely across Northern Australia (Table 5, Cowley et al., 2015). These included broodstock captured at (i) unspecified locations in Joseph Bonaparte Gulf (JBG) and supplied by 3 commercial shrimp hatcheries (SH1-SH3; Batches 1-5) or at (ii) Bramston Beach (BB), Etty Bay (EB) and Yorkeys Knob (YK) in the Innisfail-Cairns region of North Queensland (NQ) and sampled immediately after being received at CSIRO laboratories at the Queensland Bioscience Precinct (QBP; Batches 9, 10, 11) or the Bribie Island Research Centre (BIRC; Batches 13, 15, 17). Sub-adult *P. monodon* from the Gulf of Carpentaria (GoC) were sampled immediately after capture during CSIRO stock survey trawls undertaken twice-annually at specific locations (Cowley et al., 2015). Tissues were also sampled from juvenile to sub-adult *P. monodon* being reared in ponds either at BIRC (Batches 14, 16) or at a commercial shrimp farm (NQ-SF1; Batch 12) experiencing mortalities. As the diseased shrimp at NQ-SF1 displayed characteristics typical of mid-crop mortality syndrome (Spann et al., 1998), and as preliminary qPCR testing identified YHV7, pleopod tissue was sampled from 48 shrimp assessed visually as appearing normal and 48 assessed as displaying signs of morbidity.

To expedite nucleic acid extraction and PCR testing, tissues from some BIRC *P. monodon* batches were snap frozen in pelletized dry ice immediately following biopsy and stored briefly at −80 °C until processed. All others were collected into 1 mL RNAlater® solution (Thermo Fisher) at no greater than a 1:4 tissue:liquid ratio and stored short-term in a refrigerator to ensure good preservative penetration before being shipped to CSIRO-QBP where they were stored at -20 °C until processed.

2.2. Consensus RT-PCR to amplify ORF1b gene sequences from different YHV genotypes

Historically, 2 regions in the YHV ORF1b gene region have been targeted by various diagnostic PCR tests including those currently recommended in the *OIE Manual of Diagnostic Tests for Aquatic Animals* 2018 (OIE, 2018b). The upstream ORF1b gene region is targeted by the first 1-step PCR designed to detect YHV1 (OIE YHV PCR Test 1, Wongteerasupaya et al., 1997) and by a multiplex nested PCR designed to detect and differentiate YHV1 from GAV (OIE YHV PCR Test 2, Cowley et al., 2004). The downstream ORF1b gene region is targeted by a consensus nested PCR for YHV genotypes (OIE YHV PCR Test 3, Wijegoonawardane et al., 2010) as well as other consensus PCR tests used to assign YHV genotypes (Walker et al., 2001; Wijegoonawardane et al., 2008b) and to detect GAV (Cowley et al., 2000b; de la Vega et al., 2004). To generate YHV7-specific controls to assess PCR test specificity, the PCR primer pair GAV210 m:GAV211m (Table 1) was used to amplify, and subsequently clone and sequence, the upstream ORF1b gene region of representative strains of YHV7 (Mohr et al., 2015) and YHV1–6. PCR (YH30-F1m:R1m) and nested PCR (YH31-F2m:R2m) primer pairs (Mohr et al., 2015) refined from those used to assign YHV genotypes (Wijegoonawardane et al., 2008b) were also used to amplify, and subsequently clone and sequence, the downstream ORF1b gene region of representative strains of YHV1–7.

2.3. Control plasmid DNA preparation, sequencing, sequence alignment and dilution

For representative strains of YHV1–7, the ~1.2 kbp ORF1b gene region amplified using the GAV210 m:GAV211m PCR primer pair was purified using a QIAquick column (QIAGEN) and cloned into pGEM-T Easy vector using standard methods. Clones containing inserts of the expected size were identified by colony PCR (SP6:T7 primer pair), and 2–3 representatives of each were grown overnight in mini-cultures. High-purity plasmid DNA was extracted using QIAprep 2.0 Spin

Miniprep columns (QIAGEN). To sequence inserts in both orientations, 20–50 ng each pDNA was reacted with either SP6 or T7 primer and BigDye Terminator V3.1 Cycle Sequencing Kit reagents (Thermo Fisher Scientific). Amplified DNA was purified using the Agencourt CleanSEQ Clean-up Kit (Beckman Coulter) and sequenced using a 3130xl Genetic Analyzer (Thermo Fisher Scientific). Sequence chromatograms were examined and edited using Sequencher® 5.4.6 (Gene Codes Corp.) and BioEdit v7.2.6 (Hall, 1999) was used to generate and format Clustal X multiple sequence alignments.

The DNA concentration in each extract was determined by analysis of 3 x 1.5 µL aliquots on a Nanodrop 8000 UV spectrophotometer (Thermo Scientific), and DNA was diluted to a concentration of 1 µg μL^{-1} with molecular grade water and stored at −80 °C. pDNA molecular masses were calculated based on their insert sequence using OligoCalc V3.27 (Kibbe, 2007) and plasmids were diluted further in water to working stock at 2×10^{10} dsDNA molecules μL^{-1} and stored at −80 °C in 20 µL aliquots. To assess PCR test sensitivity, serial 10-fold dilutions of each pDNA were prepared in 10 ng μL^{-1} salmon sperm DNA (Roche) as a carrier to between 2.0×10^{8} and 0.2 dsDNA molecules μL^{-1}.

2.4. In vitro synthesised ssRNA control preparation and dilution

Plasmid DNA (pGEM-T-easy vector) containing a YHV7 ORF1b gene insert was linearized using Spe I, extracted with phenol:chloroform (50:50) and ethanol precipitated. Briefly, RNA was synthesised from 1 µg Spe I-cut DNA in a reaction (20 µL) containing 2 µL 10 x MEGA Script Reaction Buffer, 2 µL each NTP and 2 µL T7 RNA polymerase Enzyme Mix according to the protocol recommended in the MEGAscript® T7 Transcription Kit (Thermo Fisher Scientific). The reaction was mixed briefly, incubated at 37 °C for 3 h and synthesised RNA was purified using the MEGAclear™Kit column (Thermo Fisher Scientific) and eluted in 70 µL Elution Solution. An aliquot (0.3 µL) of the RNA was electrophoresed in agarose gel to crudely assess its yield and integrity. The RNA concentration was determined by analysis of 3 aliquots using a Nanodrop 8000 UV spectrophotometer, and 1 µL aliquots of the stock RNA were snap frozen onto the wall of microcentrifuge tubes imbedded in dry ice and stored at −80 °C. The molecular mass of the 1038 nt YHV7 synthetic ssRNA sequence (i.e. 331,536 $g\ l^{-1}$, 1 x A_{260nm} = 26.9 µg mL^{-1}) was calculated using OligoCalc. Based on this mass calculation and a ssRNA concentration of A_{260nm} = 39.86, the YHV7 RNA stock solution was calculated to possess 1.95×10^{12} RNA molecules μL^{-1}. To prepare serial 10-fold dilutions of this synthetic YHV7 RNA between 2.0×10^{8} and 0.2 RNA molecules μL^{-1}, 1 µL aliquot of stock RNA was first diluted to 2×10^{10} molecules μL^{-1} in 10 ng μL^{-1} baker's yeast tRNA (Roche) as a working stock, from which further dilutions were prepared in the same carrier RNA.

2.5. Total nucleic acid extraction and cDNA synthesis

Shrimp tissue pieces preserved in RNAlater were blotted briefly on clean paper towel to remove excess liquid before being placed into 0.6 mL Buffer RLT (QIAGEN) in wells of a 96-well pate each containing 2 glass (2 mm dia.) and 1 ceramic bead (3 mm dia.). Plates were agitated at maximum speed using a Retsch MM300 TissueLyser, supernatants were transferred to another 96-well plate and total nucleic acid (TNA = RNA + DNA) was extracted using MagJET RNA Kit reagents (Thermo Fisher Scientific) and a KingFisher™ Flex Purification System (Thermo Fisher Scientific) as described previously (Cowley et al., 2018). Before storage at −80 °C, an aliquot (1.5 µL) of each TNA eluate was analysed using a Nanodrop 8000 UV spectrophotometer to estimate RNA concentration and purity. cDNA was synthesised in a 10 µL reaction prepared using 500 ng total TNA and SensiFAST™ cDNA Synthesis Kit (Bioline) reagents incorporating an optimized mix of random hexamers and anchored oligo-dT primers as described in the reagent guide. cDNA was either amplified by PCR immediately or stored at -20 °C until used.

2.6. YHV7 TaqMan real-time qPCR

Bio-RP column-purified PCR primers (qYHV7-F1, qYHV7-F2, qYHV7-R1) and a HPLC-purified 5′-6FAM-BHQ1-3′ labelled TaqMan® probe (qYHV7-Pr1) (Bioneer Pacific, Table 1) were designed to the ORF1b gene region spanned by the GAV210 m:GAV211m PCR primer pair (Supplementary Fig. 1). The genome region was selected for test design due to the YHV7 sequence varying substantially at several locations with representative strains of YHV genotypes 1–6 and YHV8. The primer and probe sequences were selected to meet default TaqMan real-time qPCR parameters for annealing temperature and amplicon length in the Primer Express V3.0 (Thermo Fisher Scientific) and Primer3-v0.4.0 software (Koressaar and Remm, 2007; Untergasser et al., 2012). Two forward primers (qYHV7-F1, qYHV7-F2) were designed to compare their impact on test specificity and sensitivity. Reactions (20 µL) utilized SensiFAST™ Probe Lo-ROX Kit reagents (Bioline), 0.9 µM each forward and reverse primer, 0.25 µM TaqMan probe and 2 µL cDNA (equivalent to 100 ng total RNA). Reaction aliquots (5 µL) were dispensed into each of 3 wells of a 384-well real-time qPCR plate as amplification plate technical replicates. DNA was amplified using a ViiA7 Real-time PCR System (Applied Biosystems) using its default thermal cycling profile. Real-time qPCR detection sensitivity was assessed using serial 10-fold dilutions prepared to either YHV7 plasmid DNA or synthetic RNA of known copy number.

2.7. YHV7 nested PCR

YHV7-specific PCR (YHV7-F1a, YHV7-R1a) and nested PCR (YHV7-F2a, YHV7-R2a) primers were designed to an ORF1b gene sequence upstream and overlapping that targeted by the TaqMan real-time qPCR (Table 1, Supplementary Fig. 1). To promote specificity, as for the YHV7 TaqMan real-time qPCR, primers were selected to have 3′-end sequences with continuous stretches of 2–4 nucleotides that varied from those of genotypes YHV1–6. Each PCR (25 µL) contained 2 µL cDNA (equivalent to 100 ng total RNA), 1 x MyTaq™ Red Mix (Bioline), 10 pmoles each primer YHV7-F1a and YHV7-R1a and 0.125 µL MyTaq DNA Polymerase. Thermal cycling conditions were 95 °C for 1 min, 35 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 45 s followed by 72 °C for 7 min and a 20 °C hold. The nested PCR (25 µL) utilized 1 µL of the primary PCR and conditions as per the PCR except for use of primers YHV7-F2a and YHV7-R2a and a thermal cycling profile employing a 30 s extension time. Aliquots (8 µL) of each PCR or nested PCR were electrophoresed in 1.5% agarose-TAE gels containing 0.1 µL mL^{-1} SYBR Safe DNA Gel Stain to identify the 788 bp PCR and 412 bp nested PCR products. DNA was detected and gel images were captured using a Gel Doc 2000 UV transilluminator (Bio-Rad).

3. Results

3.1. YHV7 TaqMan real-time qPCR and conventional PCR test design

For the 2 ORF1b gene regions targeted by YHV/GAV diagnostic PCR tests recommended in the *OIE Manual of Diagnostic Tests for Aquatic Animals* 2018 (OIE, 2018b), available genome sequences for strains of YHV genotypes 1–8 were aligned using Clustal X to identify sequences unique to YHV7. Visual scanning of sequences aligned in the upstream ORF1b gene region, positioned just downstream of the ORF1a/1b gene overlap, identified several locations highly unique to YHV7 (Supplementary Fig. 1). Sequences at locations meeting acceptable PCR design criteria were selected for use as primers/probe in YHV7-specific conventional nested PCR and TaqMan real-time qPCR tests (Table 1).

In both tests, it was possible to include continuous stretches of 2–5 nucleotide mismatches positioned at or near to the 3′-end of either one or both PCR primers (Supplementary Fig. 1). Amplicon lengths of the conventional PCR (788 bp) and nested PCR (412 bp) components of the RT-nested PCR test and the real-time qPCR test (73 bp or 79 bp depending on the use of the F1 or F2 primer, respectively) were selected for good amplification efficiency, and in the case of the nested PCR test,

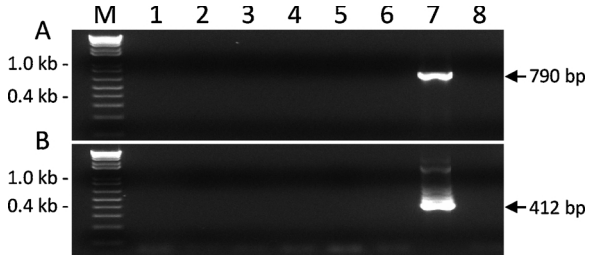

Fig. 1. Agarose gel electrophoresis of DNA amplified using the (A) PCR and (B) nested PCR components of the YHV7-specific RT-nested PCR test and 1.5×10^9 copies of plasmid DNA containing inserts of representative strains of YHV genotypes 1–7 (Lanes 1–7, respectively). An 8 µL aliquot of each 25 µL PCR was analysed. No template control reaction (Lane 8), M = 1 kb PLUS DNA ladder.

for ease of agarose gel-based detection and size discrimination of DNA products.

3.2. YHV7 conventional RT-nested PCR specificity and sensitivity

The specificity of the PCR and the nested PCR primer pairs used in the YHV7 RT-nested PCR test was assessed initially using 1.5×10^9 copies of the pDNA controls containing inserts derived from representative strains of YHV1–7 (Fig. 1). Despite the high pDNA copy number, agarose gel analysis only detected DNA products in the PCR (788 bp) and nested PCR tests (412 bp) undertaken using YHV7 pDNA. To assess the template detection limits of the YHV7 RT-nested PCR, cDNA prepared to serial 10-fold dilutions of synthetic YHV7 ssRNA of calculated copy number was amplified and analysed in an agarose gel (Fig. 2). A DNA band (788 bp) was detected in decreasing amounts down to 100 YHV7 ssRNA copies in the PCR, and in the nested PCR, a DNA band more uniform in staining intensity was clearly detected down to 10 ssRNA copies.

3.3. YHV7 TaqMan real-time qPCR test specificity and sensitivity

In the process of designing PCR primers and a [6FAM]-BHQ1-labelled probe meeting criteria for both an efficient TaqMan real-time qPCR test and YHV7 sequence specificity, 2 forward PCR primers (YHV7-qF1 and -qF2) were evaluated to identify whether one might afford better detection efficiency/specificity (Table 1, Supplementary Fig. 1). When tested using random-primed cDNA prepared to between 20 and 2×10^8 copies of a synthetic YHV7 ssRNA, the use of either forward PCR primer resulted in near-ideal PCR amplification efficiency and linearity (Table 2).

When the specific TaqMan real-time qPCR test was assessed using 1- $\times 10^8$ copies of the same YHV 1–7 pDNA controls used to assess the nested PCR test specificity, only the YHV7 pDNA generated a Ct value. qPCR data obtained using the YHV7-qF2:qR1 primer pair are shown in Table 3. In addition, when used to amplify 10-fold dilutions of the either YHV7 pDNA or ssRNA, the test reliably generated a Ct value in triplicate 5 µL reactions down to 10 copies of either template type (Tables 3 and 4). Tests performed using the YHV7-qF1:qR1 primer pair provided similar specificity for YHV7 pDNA and comparable detection sensitivities for either RNA/DNA template type.

Table 2
Ct values obtained in YHV7 real-time qPCR tests employing either the F1:R1 or F2:R1 primer pairs and cDNA prepared to serial 10-fold dilutions of synthetic YHV7 ssRNA.

YHV ssRNA copies	Ct value mean ± SD	
	F1:R1 qPCR test	F2:R1 qPCR test
20	32.45 ± 0.60	33.19 ± 0.29
200	28.86 ± 0.07	29.61 ± 0.23
2×10^3	26.11 ± 0.07	26.21 ± 0.09
2×10^4	22.95 ± 0.08	23.11 ± 0.15
2×10^5	19.41 ± 0.11	19.94 ± 0.08
2×10^6	16.46 ± 0.07	16.42 ± 0.05
2×10^7	12.56 ± 0.17	13.11 ± 0.06
2×10^8	9.30 ± 0.20	9.82 ± 0.15

Mean Ct values determined from 3 wells containing 5 µL reactions.
F1:R1 PCR: Slope = -3.273, Y intercept = 37.2, $R^2 = 0.999$, Efficiency = 102.1%, Error = 0.022.
F2:R1 PCR: Slope = -3.316, Y intercept = 37.5, $R^2 = 1.000$, Efficiency = 100.2%, Error = 0.016.

Table 3
YHV7 TaqMan real-time qPCR Ct values obtained in duplicated tests using either 10^8 to 1 copy of YHV7 pDNA or 10^8 copies of pDNA containing inserts to representative strains of YHV genotypes 1–6.

YHV genotype	YHV7 ssRNA copies	YHV7 qPCR Ct values		
		Dup 1	Dup 2	Mean ± SD
YHV7	NTC	UD	UD	–
	1	35.7	UD	–
	10	34.2	34.2	34.2 ± 0.0
	100	30.3	30.3	30.3 ± 0.1
	10^3	27.0	26.9	26.9 ± 0.1
	10^4	23.9	23.9	23.9 ± 0.0
	10^5	20.7	20.9	20.8 ± 0.2
	10^6	17.3	17.1	17.2 ± 0.2
	10^7	13.3	13.4	13.4 ± 0.1
	10^8	10.0	10.4	10.2 ± 0.3
YHV1	10^8	UD	UD	–
YHV2	10^8	UD	UD	–
YHV3	10^8	UD	UD	–
YHV4	10^8	UD	UD	–
YHV5	10^8	UD	UD	–
YHV6	10^8	UD	UD	–

NTC = no template control.
Duplicate (Dup) tests 1 and 2 (20 µL reaction volumes).
UD = Undetermined.

3.4. YHV7 PCR test application to clinical samples

As a start to identifying reference populations to assist fulfil diagnostic sensitivity and specificity and other fit-for-purpose criteria

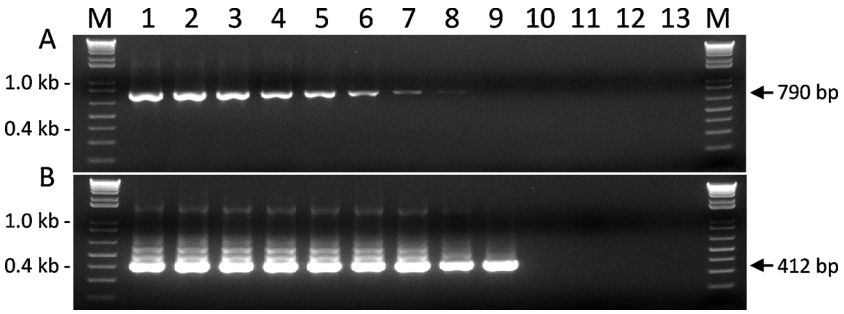

Fig. 2. Agarose gel electrophoresis of DNA amplified using the (A) YHV7 PCR test and (B) nested PCR test using cDNA prepared from serial 10-fold dilutions of YHV7 synthetic ssRNA ranging between 1×10^9 and 0.01 ssRNA copies per 25 µL reaction (Lanes 1 to 12, respectively). An 8 µL aliquot of each 25 µL reaction was analysed. No template control reaction (Lane 13), M = 1 kb PLUS DNA ladder.

Table 4
YHV7 TaqMan real-time qPCR Ct values obtained using cDNA prepared to serial 10-fold dilutions of synthetic YHV7 ssRNA.

YHV ssRNA copies	YHV qPCR Ct values			
	Rep 1	Rep 2	Rep 3	Mean ± SD
NTC	UD	UD	UD	–
0.1	UD	38.3	38.2	38.2 ± 0.1
1	UD	UD	36.8	–
10	35.5	35.3	34.7	35.2 ± 0.4
100	33.0	32.8	33.0	32.9 ± 0.1
10^3	29.2	29.3	29.4	29.3 ± 0.1
10^4	26.1	26.3	26.1	26.2 ± 0.1
10^5	22.0	22.0	22.0	22.0 ± 0.0
10^6	18.1	18.2	18.0	18.1 ± 0.1
10^7	15.1	15.0	15.0	15.0 ± 0.1
10^8	11.8	11.5	11.5	11.6 ± 0.2

NTC = no template control.
Rep1, 2 and 3 = technical replicate wells (5 µL reaction volumes).

needed to validate performance of the YHV7 real-time qPCR test (OIE, 2018a), gill or pleopod tissue was tested from 815 *P. monodon* collected between 2013 and 2019 and originating from locations spread widely across northern Australia (Table 5, Cowley et al., 2015). YHV7 RNA was detected in low to moderate amounts (Ct 37.4-20.0) at moderate prevalence (11%, 27%, 55%) among 3 batches of broodstock (Batches 1, 2 and 3; n = 81) captured at JBG and sampled in mid- and late-2013 after their use at 2 commercial shrimp hatcheries (SH1, SH2). It was also detected at low loads (Ct 37.5-33.2) in 3 (2%) of another group of 150 JBG broodstock (Batch 4) sampled at SH2 in Aug 2013, in a single shrimp trawled near Weipa among the 54 captured in the GoC in Feb 2013 (Batch 6) and in 3/104 broodstock captured in Jun/Jul 2013 at either Yorkeys Knob (YK) or Etty Bay (EB) in the Innisfail-Cairns region

Table 5
YHV7 TaqMan real-time qPCR data on gill/pleopod tissues of 815 *P. monodon* originating from various regions in Northern Australia and sampled between Feb 2013 and Apr 2019.

Shrimp					YHV7 real-time qPCR test	
Batch	Origin	Number	Sampling date	Tissue type	No. +ve	Ct range
1	JBG-SH1	20	2013-06	gill	11 (55%)	22.0 - 37.0
2		26	2013-10	pleopod	7 (27%)	23.0 - 37.1
3	JBG-SH2	35	2013-06	pleopod	4 (11%)	20.0 - 37.4
4		150	2013-08	pleopod	3 (2%)	35.8 - 37.5
5	JBG-SH3	40	2013-09	pleopod	0	–
6	GoC	54	2013-02	pleopod	1 (< 1%)	35.7
7		9	2013-06	gill	0	–
8		4	2014-02	gill	0	–
9	NQ-BB	51	2013-06	gill	0	–
10	NQ-YK	44	2013-07	pleopod	1 (2%)	35.2
11	NQ-EB	60	2013-07	pleopod	2 (3%)	33.2 - 35.1
12	NQ-SF1	96	2017-10	pleopod	96 (100%)	14.6 - 24.2
13	NQ-EB	79	2018-08	pleopod	0	–
14	NQ-BIRC	32	2018-08	pleopod	0	–
15	NQ-BB	27	2018-09	pleopod	0	–
16	NQ-BIRC	76	2018-09	pleopod	0	–
17	NQ-EB	12	2019-04	pleopod	0	–

See Materials and Methods for additional shrimp sample information; JBG = Joseph Bonaparte Gulf, GoC = Gulf of Carpentaria (various locations), NQ = North Queensland; BB = Bramston Beach; EB = Etty Bay, YK = Yorkeys Knob; H1-H3 = Shrimp hatcheries 1–3; SF1-SF2 = Shrimp farms 1 and 2, BIRC = Bribie Island Research Centre; Ct = cycle threshold.

of NQ (Batches 10, 11; Table 5). YHV7 RNA was not detected in any of a group of 40 JBG broodstock (Batch 5) provided by another hatchery (SH3) in Sep 2013, any of 3 batches of NQ broodstock (n = 118) from either EB or Bramston Beach (BB) locations and acquired by CSIRO-BIRC between Aug 2018 and Apr 2019 (Batches 13, 15, 17) or in 2 separate lines of juveniles (n = 108) derived from NQ broodstock being reared in ponds at BIRC in Aug/Sep 2018 for experimental purposes (Batches 14, 16; Table 5).

In contrast to he wild *P. monodon* samples, the YHV7 real-time qPCR test detected YHV7 RNA at moderate to high loads (Ct 24.2-14.6) in all 96 *P. monodon* (Batch 12, Table 5) sampled in Oct 2017 from a pond at a farm in North Queensland (NQ-SF1) displaying disease characteristics typical of mid-crop mortality syndrome (Spann et al., 1998). YHV7 RNA copy numbers determined from the qPCR test Ct values were compared to assess variability among the groups of 48 shrimp assessed visually as being either normal in appearance or displaying signs of morbidity (Fig. 3). *t*-test analysis of the geometric mean YHV7 RNA copy number detected in the group of shrimp assessed as being moribund ($10^{6.67}$) identified it to be significantly higher ($P = .008$) than in the group assessed as appearing normal ($10^{6.19}$).

To assess how the YHV7 RT-nested PCR test would have detected and differentiated these samples, cDNA from 4 individuals with either the lowest ($10^{4.95}$-$10^{5.37}$) or highest ($10^{7.95}$-$10^{8.16}$) YHV7 RNA amounts was amplified and analysed by agarose gel electrophoresis (Fig. 4a). A 788 bp PCR amplicon was detected with all 8 samples, but at lower relative amounts with the 4 YHV7-low RNA samples. Uniformly high

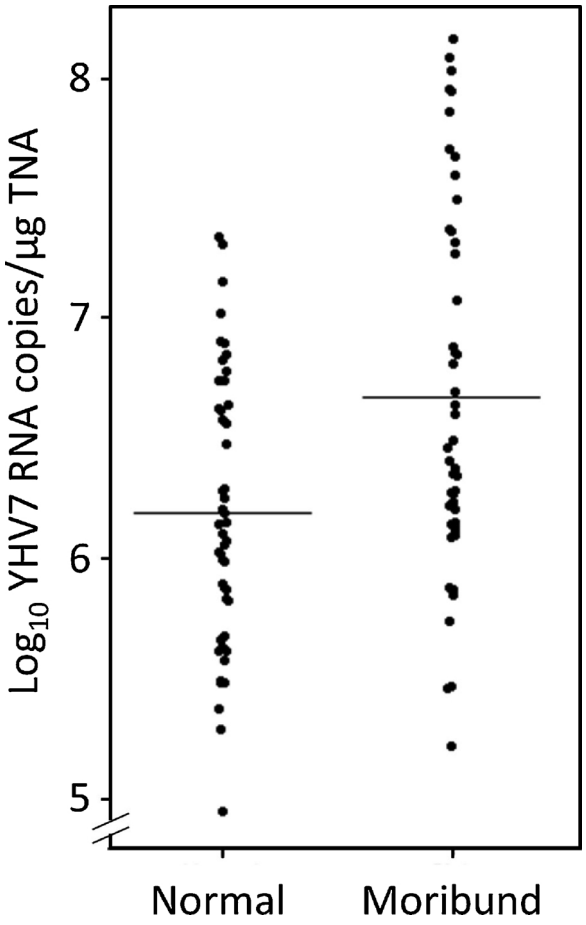

Fig. 3. Jitter plot generated using R of YHV7 RNA copy numbers determined by YHV7 TaqMan real-time qPCR test for groups of 48 *P. monodon* from a farm pond that were scored visually upon capture as either appearing normal or displaying various signs of being moribund. Lines represent geometric mean values for the cohorts assessed visually as being normal or moribund.

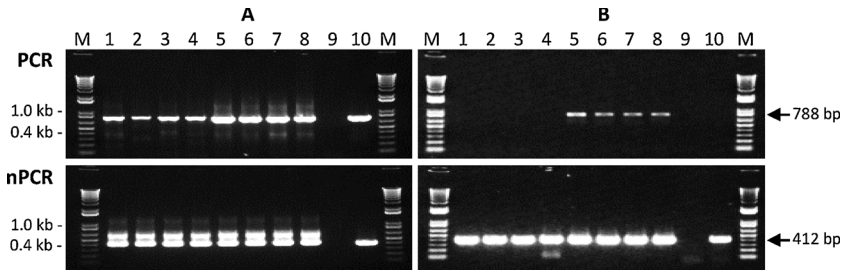

Fig. 4. Agarose gel electrophoresis of DNA amplified by the YHV7 PCR and nested PCR tests using either (A) 10 μM or (B) 5 μM primers in the PCR step of the test. The PCR tests were applied to cDNA prepared to TNA extracted from pleopods of groups of 4 farmed *P. monodon* that produced either high (Ct range 23.7–25.6) or low (Ct range 14.6–15.3) Ct values in the YHV7 TaqMan real-time qPCR (see Fig. 4). High Ct shrimp (Lanes 1–4), low Ct shrimp (lanes 5–8), no template control (Lane 9), YHV7-positive cDNA (Lane 10), M = 1 kb PLUS DNA ladder.

amounts of 412 bp nested PCR amplicon were detected with all 8 samples, but with an additional less-well resolved and slightly larger amplicon not evident in the YHV7 positive control sample. However, as this control was known to contain moderately high amounts of YHV7 RNA, the PCR was diluted 1:20 prior to its use in the nested PCR to avoid the potential for PCR artefacts caused by excessive DNA template.

As a more direct means of restricting PCR efficiency to reduce amplicon amounts added to the nested PCR, all 8 samples were re-amplified using 5 rather than 10 pmoles of each primer (Fig. 4b). As expected, this reduced primer amount markedly reduced PCR amplicon amounts to a level where none were detected with either the 4 YHV7-low RNA samples or the YHV7 positive control. Despite this, the nested PCR generated similarly high amounts of 412 bp amplicon with all 8 clinical samples and the YHV7 control, with no evidence of the slightly larger amplicon.

4. Discussion

Penaeus monodon remain the mainstay of shrimp aquaculture in Australia. As such, the detection of a unique seventh genotype of YHV (YHV7) in a cohort of *P. monodon* broodstock experiencing disease and mortalities in a commercial hatchery in Queensland following their capture from JBG in northern Australia (Cowley et al., 2015; Mohr et al., 2015) was of major concern. As a diagnostic and research tool to assist in determining the level of threat posed by this new YHV genotype, highly sensitive and specific conventional RT-nested PCR and TaqMan real-time quantitative (q)PCR tests were developed to detect and accurately quantify YHV7 RNA.

Both YHV7-specific PCR tests were designed to the upstream ORF1b gene region of 2 targeted historically by other diagnostic PCR tests and PCR tests used to assign YHV strains to a genotype (Castro-Longoria et al., 2008; Cowley et al., 1999, 2000b, 2015; Sittidilokratna et al., 2002; Soowannayan et al., 2003; Tang and Lightner, 1999; Wijegoonawardane et al., 2008a, 2008b, 2010; Mohr et al., 2015). The ORF1b gene is also a good candidate for these purposes as it encodes well-characterised non-structural protein motifs conserved among nidoviruses interspersed with more variable nucleotide sequences (Castro-Longoria et al., 2008; Cowley et al., 2000a; Sittidilokratna et al., 2002; Lauber et al., 2013). At the time the YHV7-specific PCR tests were designed, sequence data were available for strains of YHV genotypes 1-7 in both ORF1b gene regions. Of these, the upstream sequence region bounded by the GAV210 m/GAV211m PCR primer pair was selected as the most favourable target for the design of ideal nested PCR and TaqMan real-time qPCR tests based on the YHV7 sequence possessing stretches of 2–5 nucleotides at several locations that varied quite markedly from all other genotypes (Cowley et al., 2015; Mohr et al., 2015).

PCR test specificity for YHV7 is critical, particularly as *P. monodon* inhabiting eastern and northern Australia are commonly found to be infected at high prevalence with GAV (YHV genotype 2) (Spann et al., 1998; Cowley et al., 2000b, 2002b; Callinan and Jiang, 2003; Walker et al., 2001; Munro et al., 2011), and are also now known to be sometimes infected by YHV6 (Cowley et al., 2015). Due to concurrent infections with multiple YHV genotypes, PCR test specificity is also required when applying them for research purposes to better understand the geographic distribution, prevalence, host range and pathogenicity of YHV7, which are the subject of ongoing investigations (Cowley et al., 2015; N.J. Moody et al., unpublished data). Even when evaluated using high copy numbers of plasmid DNA (1×10^8 to 1.5×10^9 dsDNA copies/reaction), conventional PCR tests undertaken using either the PCR or nested PCR primers, as well as the primers and probe employed in the TaqMan real-time qPCR test, did not cross-detect sequences representative of YHV1–6. Substantial sequence differences were also evident between the PCR test primers and the ORF1b sequence reported for an eighth unique YHV genotype (YHV8) recently reported to occur in *Penaeus chinensis* and *Penaeus vannamei* farmed in China (Dong et al., 2017). Moreover, as reported in a surveillance project in which the YHV7 nested PCR and TaqMan real-time qPCR tests were deployed, neither displayed any propensity to cross-detect clinical material containing this or either of 2 other unique YHV genotypes identified recently in uncooked commodity shrimp meat imported into Australia (Cowley et al., 2015; N.J. Moody et al., unpublished data).

Using dilution series of either YHV7 circular plasmid dsDNA or synthetic ssRNA, the reliable detection sensitivity limit of the nested PCR was 10 copies, which as expected improved that of the PCR step of the test 10- to 100-fold (Haff, 1994). The use of 2-fold rather than 10-fold dilutions of plasmid DNA or ssRNA would have determined the test detection limits more accurately, and likely demonstrated them to be somewhat lower. However, the capacity to reliably detect 10 RNA templates showed the limits of detection of the YHV7 nested PCR test to be comparable to those of conventional and real-time qPCR tests designed to either detect or co-detect YHV1 and GAV (Wongteerasupaya et al., 1997; Cowley et al., 2000a,b, 2004; de la Vega et al., 2004)

While preliminary in nature relative to the plethora of considerations in validating a new diagnostic assay for a particular purpose (OIE, 2018a), the diagnostic usefulness of the YHV7 TaqMan real-time qPCR was assessed here for the purposes of (i) determining the prevalence of this YHV genotype in geographically disparate populations of *P. monodon* (Cowley et al., 2015) and (ii) associating elevated viral loads with shrimp displaying overt signs of disease. Real-time qPCR data showed marked prevalence variations among wild broodstock captured at locations in the JBG, GoC and Innisfail-Cairns regions of northern Australia and will thus be useful in guiding the Australian *P. monodon* farming industry to locations such as the GoC and NQ where YHV7 was either not detected or detected at low prevalence. Use of the test to rigorously screen broodstock cohorts before their use would also be valuable in selecting individuals to generate YHV7-free progeny for either (i) reducing disease risks during farm rearing or (ii) establishing/augmenting virus-free breeding populations (Preston et al., 2009).

The capacity of the YHV7 qPCR test to accurately quantify viral RNA loads was demonstrated by testing pleopods sampled from groups of overtly normal looking or moribund *P. monodon* selected from a pond in which acute YHV7 infection was suspected to be causing mortality. YHV7 qPCR data substantiated mean YHV7 RNA loads to be significantly higher ($P = .008$) in the shrimp assigned visually to be displaying signs of morbidity. These data were somewhat expected and support findings with YHV1 and GAV (Cowley et al., 2000a,b; Munro et al., 2011; Sellars et al., 2011) in which viral RNA loads are commonly used as a *de facto* measure of infection severity. As all shrimp were YHV7-positive, the diagnostic capabilities of the conventional YHV7 nested PCR were only demonstrated with a small number of shrimp

possessing the lowest and highest YHV7 loads as determined using the YHV7 qPCR. While the YHV RNA loads present in these groups of shrimp generated differential amounts of 788 bp PCR amplicon, all generated uniformly high amounts of 412 bp nested PCR amplicon. The nested PCR also generated a secondary amplicon for all 8 samples tested that was not apparent in the control test in which the PCR was diluted 1:20 prior to nested PCR. This suggested that its occurrence was due to excessive amounts of PCR amplicon being transferred to the nested PCR under the standard method conditions used. Confirming this to be the cause, the nested PCR artefact disappeared when the PCR primer concentration used in the standard method was halved to artificially, and unacceptably in terms of test sensitivity, reduce PCR amplicon yields. However, if there is a desire to confirm a putative specific PCR diagnosis of YHV7 by running a nested PCR (Haff, 1994), particularly when a PCR amplicon is clearly evident, diluting the PCR 1:20 to reduce excessive template amounts is recommended to avoid the potential for artefactual nested PCR products confusing a diagnosis.

Based on sequences of YHV genotypes available at the time, nested PCR and SYBR-Green real-time qPCR tests have been developed to cross-detect and identify new genotypes (Walker et al., 2001; Wijegoonawardane et al., 2008a, 2008b, 2009, 2010; Mohr et al., 2015). By serendipity, a multiplexed nested PCR test designed to co-amplify and differentiate YHV1 from GAV (Cowley et al., 2004), and its commercialized iteration as the IQ2000™ YHV/GAV Detection and Typing System (GeneReach Biotechnology Corp.), also cross-amplify several other genotypes (Cowley et al., 2015) which has resulted in unknown genotypes such as YHV5 and YHV8 being discovered (Soowannayan et al., 2003; Dong et al., 2017). Other tests such as the conventional PCR for YHV1 (Wongteerasupaya et al., 1997, Cowley et al., 2004) and real-time RT-PCRs designed to detect GAV (de la Vega et al., 2004) also cross-detect other genotypes at varying sensitivities (Cowley et al., 2015). Based on these deficiencies and YHV existing as a complex of related genotypes likely to have evolved in different directions in geographically isolated shrimp populations (Walker et al., 2001; Cowley et al., 2002a,b), data obtained with most existing PCR tests need to be interpreted with care, particularly in cases when knowing what genotype was detected is critical. While the research reported here and elsewhere has made inroads into developing conventional and real-time qPCR tests that provide increased specificity for detecting YHV1 and YHV7 (Cowley et al., 2015), further effort is required to devise tests providing specificity for each of the increasing diversity of YHV genotypes (Dong et al., 2017; N.J. Moody et al., unpublished data). Development of consensus tests designed to cross-detect different genotypes (Wijegoonawardane et al., 2008a, 2008b) such as that using the 210 m/211m PCR primer pair described here, combined with amplicon sequence analyse, also needs to be considered.

The sensitive and specific YHV7 real-time qPCR test described here is currently being deployed to detect YHV7 in wild and domesticated *P. monodon* broodstock prior to their use in hatcheries to assess and minimize the risks of it being transmitted vertically to progeny, as demonstrated to occur with other genotypes such as GAV (Cowley et al., 2002b). The test is also being deployed as a high-throughput diagnostic assay to detect and quantify YHV7 loads in farmed *P. monodon* experiencing disease and mortalities. Moreover, the specificity and sensitivity of the test are being exploited in surveys to identify the presence and prevalence of YHV7 in wild populations of *P. monodon* (Cowley et al., 2015), and in Australia, its inclusion in suites of PCR tests will be invaluable in selecting and maintaining specific-pathogen-free breeding lines of this valuable aquaculture species.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.113689.

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