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New yellow head virus genotype (YHV7) in giant tiger shrimp *Penaeus monodon* indigenous to northern Australia

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ABSTRACT: In 2012, giant tiger shrimp *Penaeus monodon* originally sourced from Joseph Bonaparte Gulf in northern Australia were examined in an attempt to identify the cause of elevated mortalities among broodstock at a Queensland hatchery. Nucleic acid extracted from ethanol-fixed gills of 3 individual shrimp tested positive using the OIE YHV Protocol 2 RT-PCR designed to differentiate yellow head virus (YHV1) from gill-associated virus (GAV, synonymous with YHV2) and the OIE YHV Protocol 3 RT-nested PCR designed for consensus detection of YHV genotypes. Sequence analysis of the 794 bp (Protocol 2) and 359 bp (Protocol 3) amplicons from 2 distinct regions of ORF1b showed that the yellow-head-complex virus detected was novel when compared with Genotypes 1 to 6. Nucleotide identity on the Protocol 2 and Protocol 3 ORF1b sequences was highest with the highly pathogenic YHV1 genotype (81 and 87%, respectively) that emerged in *P. monodon* in Thailand and lower with GAV (78 and 82%, respectively) that is enzootic to *P. monodon* inhabiting eastern Australia. Comparison of a longer (725 bp) ORF1b sequence, spanning the Protocol 3 region and amplified using a modified YH30/31 RT-nPCR, provided further phylogenetic evidence for the virus being distinct from the 6 described YHV genotypes. The virus represents a unique seventh YHV genotype (YHV7). Despite the mortalities observed, the role of YHV7 remains unknown.

KEY WORDS: Yellow head virus · Genotype 7 · *Penaeus monodon* · Australia

INTRODUCTION

Yellow head virus (YHV) emerged in the early 1990s as the cause of mass mortalities of giant tiger shrimp *Penaeus monodon* farmed in Thailand (Chantanachookin et al. 1993). In Thailand YHV continues to cause farm production losses and has become established as a pathogen in farmed Pacific white shrimp *P. vannamei* (Senapin et al. 2010). In addition, YHV has been reported from most shrimp farming regions where *P. monodon* occur and has also emerged in Pacific blue shrimp *P. stylirostris* in the Gulf of California (Castro-Longoria et al. 2008). While YHV is exotic to Australia, a closely related genotypic variant, gill-associated virus (GAV, synonymous with YHV2), was discovered not long after the discovery of YHV (Spann et al. 1997, Cowley et al. 1999). GAV is enzootic and can occur 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). GAV has also been detected in shrimp farmed in Vietnam and Thailand (Wijegoonawardane et al. 2008a).

GAV is designated as the type species of the genus *Okavirus*, family *Roniviridae* in the order *Nidovirales* (Walker et al. 2005). ORF1b sequence comparisons of yellow head–related viruses detected in *P. monodon* collected across the Indo-Pacific region between 1997 and 2004 identified 6 distinct genotypes (Wijegoonawardane et al. 2008a). Except for YHV (YHV1)

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and GAV (YHV2), genotypes YHV3 to YHV6 were detected in healthy *P. monodon*. The latter genotypes were described based on both the ORF1b phylogeny and their geographic location (e.g. YHV3 in Thailand and Vietnam, YHV4 in India, YHV5 in Malaysia, Thailand and the Philippines, and YHV6 in Mozambique). YHV1 types occurring in Thailand have also been assigned to subtypes YHV1a and YHV1b based on the latter possessing a 54 amino acid deletion in the N-terminal region of the ORF3 coding sequence, although both genotypes retain similar virulence in *P. vannamei* (Sittidilokratna et al. 2009, Senapin et al. 2010).

P. monodon is the primary shrimp species farmed in Australia, and, in recent years, substantial progress has been made in the domestication and selective breeding of this species (Preston et al. 2010). However, most hatcheries supplying *P. monodon* seedstock for aquaculture remain reliant on wild-caught broodstock. Until recently, the majority of wild broodstocks were sourced from the Cairns-Innisfail region of North Queensland. However, the high prevalence of GAV in the region has led to hatcheries sourcing broodstock from increasingly remote locations in northern Australia.

In 2012, a Queensland hatchery reported elevated mortalities in *P. monodon* broodstock sourced from Joseph Bonaparte Gulf in northern Australia. This report describes the molecular assays which led to the identification of a novel yellow-head-complex virus in samples from diseased animals. The role of the novel yellow-head-complex virus in the disease episode is unknown.

MATERIALS AND METHODS

Shrimp sample collection and processing

In November 2012, a batch of 414 wild *Penaeus monodon* broodstock were caught by net and trawl in Joseph Bonaparte Gulf in northern Australia and transported to a commercial hatchery in North Queensland. Upon arrival, the shrimp were bathed in a prophylactic solution containing formalin and povidine-iodine before being placed into concrete raceways. Mortalities began to occur from Day 1 post-stocking, increasing progressively to 81.4% by Day 12.

Due to the high mortality rate, tissues for histology, bacteriology and PCR were sampled from 3 moribund broodstocks by the Queensland Department of Agriculture, Fisheries and Forestry (QDAFF). Cephalothoraxes were fixed in Davidson's AFA fixative, processed for histology, and tissue sections were stained with either haematoxylin & eosin or Brown & Brenn's Gram stain using standard procedures (Lightner 1996). Preliminary analysis suggested the presence of a yellow-head-complex virus.

Three samples of gill and epidermis, fixed in ethanol, were submitted to the CSIRO AAHL Fish Diseases Laboratory, Geelong, Victoria, for confirmatory testing. Samples were homogenised in 600 µl AVL buffer in MagNA Lyser green bead tubes using the MagNA Lyser (Roche) and clarified by centrifugation at 10 000 × *g* for 5 min. RNA was extracted from 140 µl using the QIAmp Viral RNA Mini Kit (QIAGEN) and eluted in a final volume of 60 µl with AVE buffer.

PCR tests

The samples were initially tested using 3 PCR protocols as described in OIE (2014). The OIE YHV Protocol 2 multiplex reverse transcriptase-nested PCR (RT-nPCR) was performed as 2 separate RT-nPCR assays, one specific for GAV and the other for YHV1. An additional RT-nPCR, using the YH30-F2/R2 RT-PCR and YH31-F2/R2 nPCR primers targeting ORF1b (Wijegoonawardane et al. 2008a) encompassing the OIE YHV Protocol 3 RT-PCR region was also used, with modified primers. The 4 modified YH30m/YH31m RT-nPCR primers had additional redundancy to attempt the amplification of more divergent yellow-head-complex viruses. Primer sequences and thermal cycling conditions used in each PCR test are detailed in Table 1.

For each RT-PCR, 2 µl RNA was added to 23 µl reaction mixture containing 12.5 µl 2× reaction mix, 1 µl Superscript® III RT/Platinum® Taq mix (Invitrogen), 180 nM of each primer and molecular grade water. For each nPCR, 2 µl of the RT-PCR was added to 23 µl reaction mix containing 12.5 µl HotStarTaq master mix (QIAGEN), 360 nM of each primer and molecular grade water. Positive control nucleic acids from YHV1 and GAV were included in each assay. To confirm the success of the extraction and the absence of PCR inhibitors, each sample was tested using the OIE Decapod PCR targeted to shrimp genomic 18S rRNA (Lo et al. 1996).

DNA sequencing and phylogenetic analyses

Amplicons resolved in SYBR safe-stained 1.5% agarose gels were excised and purified with the

QIAquick gel extraction kit (QIAGEN). Each amplicon was Sanger-sequenced using both the forward and reverse primers and the BigDye® Terminator v3.1 Cycle Sequencing reagent (Life Technologies) by direct-product sequencing. Geneious software (Biomatters) was used to generate consensus sequences, multiple sequence alignments and calculate pair-wise distances. Phylogenetic analysis using the neighbor-joining method with a bootstrap consensus tree being inferred from 10 000 replicates was conducted using MEGA5.1 software (Tamura et al. 2011).

RESULTS

Histology and bacteriology

In all 3 moribund broodstocks that were examined by histology, lesions included neuropathy of the eye, antennae, ventral nerve cord and segmental ganglia of pereiopods and retinitis (data not shown). The lesions included lymphoid organ spheroids indicative of virus infection and were typical of those described by Callinan et al. (2003) for acute GAV infection. Bacterial culture of haemolymph produced

Table 1. Molecular assays, primers and thermal cycling conditions used in this study. ORF1b region numbering based on the yellow head virus 1 (YHV1) genome, EU487200.1

PCR Assay	Primer	Sequence (5′–3′)	Thermal cycling conditions No.	Temp.	Time	Amplicon size (bp)	ORF1b region	Reference
OIE YHV Protocol 1								
RT-PCR	10F	CCGCTAATTTCAAAAACTACG	1×	50°C	30 min	135	13830–13964	Wongteerasupaya et al. (1997)
	144R	AAGGTGTTATGTCGAGGAAGT		94°C	2 min			
			40×	94°C	30 s			
				58°C	45 s			
				68°C	45 s			
			1×	68°C	7 min			
OIE YHV Protocol 2								
RT-PCR	GY1	GACATCACTCCAGACAACATCTG	1×	50°C	30 min	794	12848–13641	Cowley et al. (2004)
	GY4	GTGAAGTCCATGTGTGTGAGACG		95°C	2 min			
			35×	95°C	30 s			
				66°C	30 s			
				68°C	45 s			
			1×	68°C	7 min			
GAV nPCR	GY2	CATCTGTCCAGAAGGCGTCTATGA	1×	95°C	15 min	406	12865–13270	Cowley et al. (2004)
	G6	GTAGTAGAGACGAGTGACACCTAT	35×	95°C	30 s			
				66°C	30 s			
				72°C	45 s			
			1×	72°C	7 min			
YHV1 nPCR	GY2	As above	As per OIE YHV			277	12865–13141	Cowley et al. (2004)
	Y3	ACGCTCTGTGACAAGCATGAAGTT	Protocol 2 GAV nPCR					
OIE YHV Protocol 3								
RT-PCR	YC-F1a	ATCGTCGTCAGCTACCGCAATACTGC	1×	50°C	55 min	359	17441–17799	Wijegoonawardane et al. (2008b)
	YC-F1b	ATCGTCGTCAGYTAYCGTAACACCGC		95°C	2 min			
	YC-R1a	TCTTCRCGTGTGAACACYTTCTTRGC	35×	94°C	45 s			
	YC-R1b	TCTGCGTGGGTGAACACCTTCTTGGC		60°C	45 s			
				68°C	45 s			
			1×	68°C	7 min			
nPCR	YC-F2a	CGCTTCCAATGTATCTGYATGCACCA	1×	95°C	15 min	147	17513–17659	Wijegoonawardane et al. (2008b)
	YC-F2b	CGCTTYCARTGTATCTGCATGCACCA	35×	95°C	30 s			
	YC-R2a	RTCDGTGTACATGTTTGAGAGTTTGTT		66°C	30 s			
	YC-R2b	GTCAGTGTACATATTGGAGAGTTTRTT		72°C	45 s			
			1×	72°C	7 min			
YH30m/31m								
RT-PCR	YH30-F1m	TACCAYTCAAACATCATYAAYAAYCAYCA	1×	50°C	30 min	1001	17084–18084	Modified from Wijegoonawardane et al. (2008a)
	YH30-R1m	GAGATGATYTGRTKCTTRAAYTTCTGRAA		94°C	2 min			
			35×	94°C	45 s			
				55°C	45 s			
				68°C	60 s			
			1×	68°C	7 min			
nPCR	YH31-F2m	CTCARATCCATGCMATYTGGGARTCHTC	1×	95°C	15 min	721	17277–17997	Modified from Wijegoonawardane et al. (2008a)
	YH31-R2m	AGT TTG GCR CGR ATR TTR GTR AGR AT	35×	94°C	45 s			
				55°C	45 s			
				72°C	60 s			
			1×	72°C	7 min			

Table 2. Performance of OIE (World Organisation for Animal Health) YHV diagnostic RT-PCR and RT-nPCR assays on RNA extracted from *Penaeus monodon* samples. GAV: gill-associated virus; (+/–) positive/negative results, respectively

PCR assay	Target	Sample 1	Sample 2	Sample 3
OIE YHV Protocol 1 RT-PCR	YHV1	–	–	–
OIE YHV Protocol 2 RT-PCR	YHV1/GAV	+	+	+
OIE YHV Protocol 2 GAV nPCR	GAV	–	–	+
OIE YHV Protocol 2 YHV1 nPCR	YHV1	–	–	–
OIE YHV Protocol 3 RT-PCR	YHV1-YHV6	+	+	+
OIE YHV Protocol 3 nPCR	YHV1-YHV6	+	+	+

Table 3. Nucleotide identity of the Sample 1 OIE YHV Protocol 2 RT-PCR, ORF1b sequence (KP738160), in comparison to available GenBank sequences

Genotype	GenBank accession no.	Country	Percent identity
YHV1	FJ848673	Thailand	80.4
YHV1	FJ848674	Thailand	81.2
YHV1	EU487200	Thailand	81.0
YHV1	FJ848675	Thailand	79.8
YHV1	AF148846	Thailand	80.4
YHV1	EU977578	Thailand	80.4
YHV1	FJ627274	Thailand	79.7
GAV	AF227196	Australia	77.6
YHV5	EU170438	Thailand	79.7
Unassigned	KF278563	China	78.1

pure growth of *Vibrio harveyi*, and the presence of a bacterial septicaemia was supported by the observation of multiple melanised granulomas, haemocyte aggregations and haemocyte nests (data not shown).

Molecular analysis

Three OIE YHV PCR protocols were performed to determine whether a yellow-head-complex virus was associated with the mortality. The OIE YHV Protocol 1 RT-PCR, specific for YHV1, was negative for all samples (Table 2). The OIE YHV Protocol 2 RT-PCR (Protocol 2) yielded an amplicon of 794 bp for all samples, but the YHV nPCR, specific for YHV1, was negative. The GAV nPCR produced a faint amplicon of 359 bp, indicative of GAV, in Sample 3 (Table 2). The OIE YHV Protocol 3 RT-nPCR (Protocol 3), capable of amplifying all 6 YHV genotypes, generated amplicons for all 3 samples in both the RT-PCR and nPCR of approximately 360 and 150 bp, respectively (Table 2).

The sequence of Sample 1 obtained from the Protocol 2 RT-PCR amplicon (748 bp primer trimmed, KP738160), yielded 10 BLAST hits with complete coverage for the amplified ORF1b region (Table 3). Seven Thai YHV1 sequences available in GenBank were most similar to the Sample 1 sequence, but only shared 81.2% or less identity. In contrast, the Australian GAV sequence (AF227196) was the least similar at 77.6% identity. A yellow-head-complex virus of a new genotype from China (KF278563) and the YHV5 sequence from Thailand (EU170438) also shared <80% identity. However, due to the lack of publicly available YHV3, YHV4 and YHV6 sequences in this ORF1b region, it was not possible to determine the YHV genotype detected.

The Protocol 3 RT-PCR amplicons (307 bp primer trimmed) for all 3 samples were sequenced (KP738161, KP738162 and KP738163) and found to be identical except for 1 nucleotide difference in Sample 1. Again, the highest identity (87.6%) to either sample was with YHV1 when comparing the same ORF1b region for representatives of YHV Genotypes 1 to 6 (Table 4). The YHV4 genotype had the lowest identity (80.1%) when compared to Sample 1. A sequence for the new Chinese YHV genotype (Liu et al. 2014) for the ORF1b region amplified by Protocol 3 RT-PCR was not available from GenBank. Based on the low sequence identity in 2 distinct ORF1b regions amplified by the Protocol 2 RT-PCR and Protocol 3 RT-PCR, the Queensland hatchery samples appeared to contain a previously unknown YHV genotype.

Table 4. Percent nucleotide identity of the OIE YHV Protocol 3 RT-PCR, ORF1b region, for Samples 1 (KP738161), 2 (KP738162) and 3 (KP738163), compared to representative strains of YHV Genotypes 1 to 6

GenBank accession no.	Strain	Country	Genotype	Percent identity Sample 1	Samples 2 & 3
EU784982	THA-03-D1	Thailand	YHV1	87.3	87.6
EU785029	AUS-00-H2	Australia	GAV	81.4	81.8
EU784996	VNM-02-H278	Vietnam	YHV3	81.8	82.1
EU785005	IND-02-H5	India	YHV4	80.1	80.5
EU785003	MYS-03-H4	Malaysia	YHV5	86.0	86.3
EU785036	MOZ-04-H11	Mozambique	YHV6	81.1	81.4

Table 5. Nucleotide (upper right) and amino acid (lower left) pair-wise comparison of the YH30m/31m RT-nPCR, ORF1b region of YHV7 (Sample 2—KP738164) with representative YHV1 (EU784982), GAV (EU785029), YHV3 (EU784996), YHV4 (EU785005), YHV5 (EU785003) and YHV6 (EU785036) sequences

	YHV1	GAV	YHV3	YHV4	YHV5	YHV6	YHV7
YHV1	–	81.8	82.8	80.8	84.3	81.7	85.5
GAV	94.5	–	93.2	82.1	84.1	96.5	82.9
YHV3	95.0	97.7	–	82.0	84.7	92.9	83.1
YHV4	91.4	93.2	93.2	–	84.7	82.5	81.7
YHV5	94.5	96.8	95.9	94.5	–	84.3	85.9
YHV6	94.5	100.0	97.7	93.2	96.8	–	83.1
YHV7	93.2	95.0	93.6	90.9	93.6	95.0	–

To further characterise this novel YHV genotype, a longer region of ORF1b encompassing the Protocol 3 RT-PCR region was amplified using the YH30m/31m RT-nPCR. The 670 bp primer-trimmed sequence from Sample 2 (KP738164) was compared with ORF1b sequences from 69 strains representing each of the yellow-head-complex virus genotypes. Sample 2 clustered phylogenetically most closely with the YHV1 genotype cluster (Fig. 1), although identity was approximately 86% with both YHV1 and YHV5 (Table 5). Translation of the ORF1b sequences revealed the novel genotype had 95% identity with GAV (YHV2) and YHV6 and only a 93.2% identity to YHV1 (Table 5).

DISCUSSION

A new yellow-head-complex genotype, YHV7, has been identified based on low nucleotide identity (<88%) and phylogenetic comparisons of the ORF1b region with the 6 previously characterised YHV genotypes. Since the discovery of GAV, this work describes the only other detection of a yellow-head-complex virus in Australia. The YHV7 virus was detected in diseased *Penaeus monodon* broodstock, but the source could be shrimp from either the Joseph Bonaparte Gulf or the Queensland hatchery. The roles of YHV7 and the bacterial infection contributing to the poor health and mortality observed in *P. monodon* are unknown.

The Protocol 2 RT-nPCR is a sensitive multiplex nested RT-PCR designed to differentiate YHV1 from GAV (YHV2), but a lack of specificity in the nested step has previously been identified with this protocol (OIE 2014). The detection of an RT-PCR amplicon and no nested PCR amplicons, as was the case for YHV7, has occurred previously with a YHV1b/YHV5

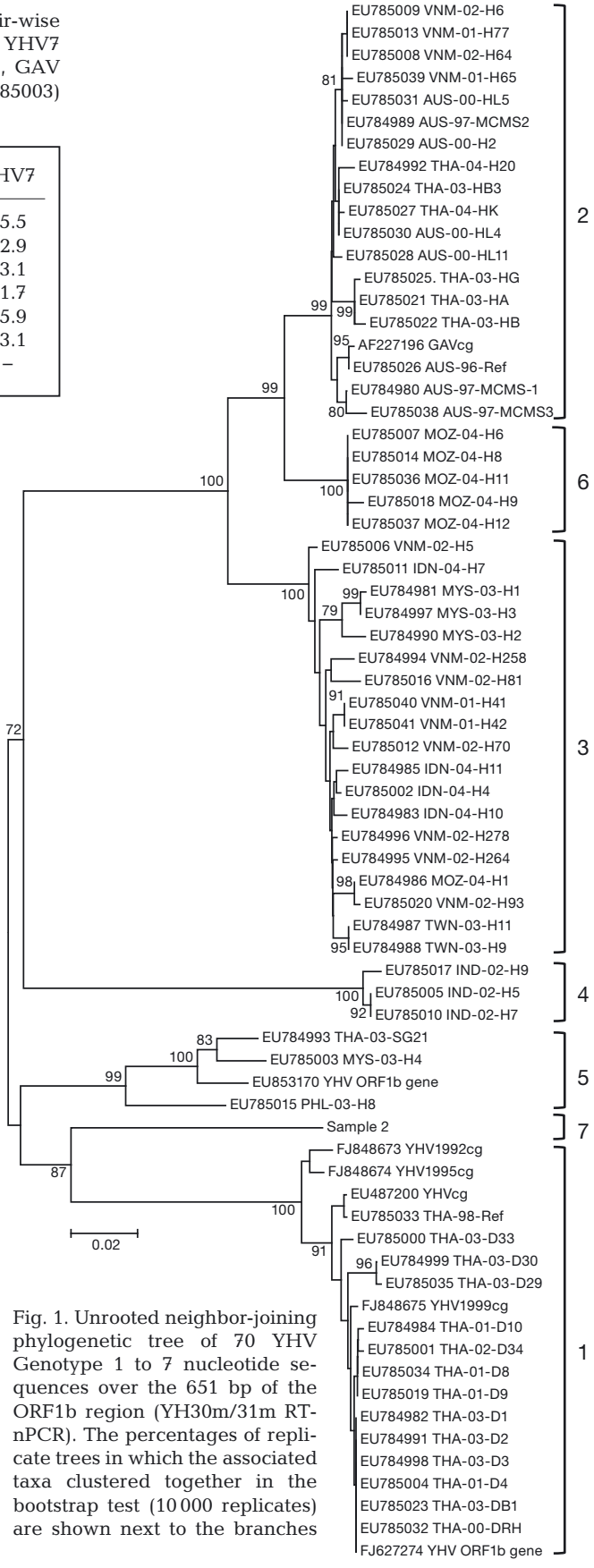

Fig. 1. Unrooted neighbor-joining phylogenetic tree of 70 YHV Genotype 1 to 7 nucleotide sequences over the 651 bp of the ORF1b region (YH30m/31m RT-nPCR). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (10 000 replicates) are shown next to the branches

recombinant virus (Gangnonngiw et al. 2009). A similar result has also been observed for an, as yet, unassigned yellow-head-complex virus detected in several provinces of China (Liu et al. 2014). However, our analysis demonstrated that YHV7 was distinct from the Chinese isolate (Table 3). In contrast, the Protocol 3 RT-nPCR was able to generate amplicons for YHV7 in both PCR steps, expanding the number of genotypes detected by this consensus assay to 7.

To assist the Australian shrimp aquaculture industry in assessing what risks YHV7 might pose, research is currently underway to determine its prevalence in wild *P. monodon* in regions where broodstock are commonly captured. Transmission trials to determine the pathogenicity of YHV7 in *P. monodon* are also required. However, to avoid confounding factors, GAV-free *P. monodon* would need to be sourced for such experiments. Research is also underway to sequence additional YHV7 isolates to enable greater understanding of the relationship of YHV7 with the previously characterised okavirus isolates.

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