A virulent isolate of yellow head nidovirus contains a deformed envelope glycoprotein gp116

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Introduction

Yellow head virus (YHV) is a highly virulent pathogen of penaeid shrimp (Penaeus monodon) which is farmed in many countries in the Indo-Pacific region. It causes a systemic infection of tissues of ectodermal and mesodermal origin that can result in total crop loss within several days of the first signs of disease in a pond (Limsuwan, 1991; Chantanachookin et al., 1993). YHV is a (+) ssRNA virus that has been classified in the genus Okavirus, family Roniviridae, order Nidovirales (Walker et al., 2005). The 26,662 nt genome of the prototype strain of YHV (Chachoengsao, Thailand, 1998) contains 4 long open reading frames. ORF1a and ORF1b overlap and encode non-structural proteins that are involved in replication and transcription (Sittidilokratna et al., 2002, 2008). Polyprotein 1a (pp1a) contains papain-like and 3C-like protease domains. Polyprotein 1ab (pp1ab) is expressed as a result of a −1 ribosomal frame-shift in the overlap region and encodes multiple enzymes of the replication complex including ‘SDD’ RNA-dependent RNA polymerase, helicase, metal-ion-binding, endoribonuclease, uridyly-specific endoribonuclease and ribose-2′-O-methyl transferase domains. ORF2 encodes the nucleoprotein (p20) (Sittidilokratna et al., 2006). ORF3 also encodes a polyprotein (pp3) that is processed to generate envelope glycoproteins (gp116 and gp64) and an N-terminal triple-membrane spanning protein of unknown function (Jitrapakdee et al., 2003). Proteolytic processing of pp3 occurs at signal peptidase type 1 consensus motifs located
adjacent to two of six hydrophobic domains that appear to be membrane-spanning (Jitrapakdee et al., 2003).Envelope glycoproteins gp116 and gp64 and the nucleoprotein p20 are the only major structural proteins in the rod-shaped (∼45 nm × ∼180 nm), enveloped YHV virions (Nadala et al., 1997; Wang and Chang, 2000; Jitrapakdee et al., 2003).

YHV is one of several closely related viruses that have been detected in *P. monodon* shrimp in the Indo-Pacific region. Gill-associated virus (GAV) occurs commonly in healthy shrimp from Australia, Thailand and Vietnam and has been associated in Australia with a slowly progressing disease named mid-crop mortality syndrome (MCMS) (Spann et al., 1997; Walker et al., 2001; Wijegoonawardane et al., 2008a). The 26,235 nt GAV genome is similar to that of YHV but includes an additional small open reading frame (ORF4) in the 3′-terminal region that may not be functional (Cowley et al., 2000, 2004; Cowley and Walker, 2002). The YHV and GAV genomes share overall nucleotide sequence identity of ∼79% and amino acid sequence identities that range from ∼73% in gp116 to ∼84% in pp1ab (Sittidilokratna et al., 2008). At least four other genotypes in the YHV complex occur commonly in healthy *P. monodon* from Mozambique in the west to Taiwan in the east and as far south as Indonesia, but none has been implicated in disease (Soowannayan et al., 2003; Wijegoonawardane et al., 2008a). Only YHV (genotype 1) has been associated with yellow head disease.

YHV infectivity for shrimp primary lymphoid organ cell cultures can be neutralised by antibody to the envelope glycoprotein gp116, but not by antibody to gp64 (Assavalapsakul et al., 2005). Glycoprotein gp116 has been shown to bind to a 65 kDa protein in lymphoid organ cells which is assumed to be the cell receptor (Assavalapsakul et al., 2005). It has also been reported that gp116 is preferentially suppressed in shrimp that survive lethal challenge with YHV (Long-yant et al., 2006; Chaivisuthangkura et al., 2008). In this paper, we describe an isolate of YHV from a disease outbreak in farmed *Penaeus vannamei* shrimp in Ratchaburi Province, Thailand in February 2006. The isolate is shown to be virulent in experimental infections but, unlike the prototype Chachoengsao/1998 isolate, it is not neutralised by polyclonal antiserum to either gp116 or gp64. Sequence analysis of the ORF3 gene indicates that this isolate, and two other isolates from disease outbreaks in *P. monodon* in Thailand, contain a deletion eliminating six cysteine residues at the N-terminus of gp116 that appears to cause a significant deformation in the structure of the glycoprotein.

**Results**

**SDS-PAGE and immunoblot analysis using gp116 and gp64 antisera**

The Chachoengsao/1998 YHV reference strain (THA-98-Ref) that was isolated from diseased *P. monodon* in 1998, and the Ratchaburi YHV isolate (THA-06-D1) from diseased *P. vannamei* in 2006 were purified from the hemolymph of infected shrimp and analysed by SDS-PAGE and immunoblotting (Fig. 1). In the Chachoengsao/1998 strain, Coomassie-staining of the p20 nucleoprotein and gp116 and gp64 envelope glycoproteins suggested their presence in similar molar abundance, as noted previously (Jitrapakdee et al., 2003). However, in the Ratchaburi/2006 isolate, gp116 was smaller and present in relatively lower abundance than gp64. Immunoblotting was conducted using polyclonal rabbit antiserum to recombinant gp116 and gp64 purified from baculovirus-infected insect cells as well as a mixed pool of mouse monoclonal antibodies to gp116 (V3-2B), gp64 (Y18) and p20 (Y19). Recombinant proteins used to prepare polyclonal antisera were derived from the Chachoengsao/1998 YHV isolate. In each preparation of purified virus, anti-gp64 polyclonal serum detected gp64 and a number of smaller proteins.

![Fig. 1. SDS-PAGE and immunoblot analysis of purified virus preparations of YHV isolates (a) Cachoengsao/1998 and (b) Ratchaburi/2006. Gels were either fixed and stained with Coomassie brilliant blue R-250 or transferred to PDVF membranes for immunoblotting with pre-immune rabbit serum, anti-gp64 serum, anti-gp116 serum (1/10^5 dilution) or a mixture of mouse monoclonal antibodies (1/5000 dilution) to YHV gp64 (MAb Y18), gp116 (MAb V3-2B) or p20 (Y19).](image-url)
that are likely to be proteolytic cleavage products. In contrast, anti-gp116 polyclonal serum detected gp116 in the Chachoengsao/1998 isolate but reacted very poorly with the smaller glycoprotein band in the Ratchaburi/2006 isolate. The mixed pool of monoclonal antibodies detected all three viral structural proteins in the Chachoengsao/1998 but detected only gp64 and p20 in Ratchaburi/2006. The smaller, less abundant gp116 (designated gp116Δ) in the Ratchaburi/2006 isolate was not detected by the gp116-specific monoclonal antibody.

Comparative infectivity titration

Bioassays were conducted in SPF *P. vannamei* to determine the LD₅₀ of filtered whole cephalothorax extracts prepared from moribund shrimp infected experimentally with YHV isolates Chachoengsao/1998 or Ratchaburi/2006. Mortalities that occurred in groups of 12 shrimp injected with 10-fold serial dilutions of each inoculum are shown in Fig. 2. For each isolate, the incubation period was 2 days and mortalities occurred until day 6 post-infection. For isolate Chachoengsao/1998, the minimum lethal dose, which resulted in 100% cumulative mortality at day 6, was a 10⁻¹⁰ dilution of the stock inoculum. For isolate Ratchaburi/2006, the minimum lethal dose, which also resulted in 100% cumulative mortality at day 6, was 10⁻⁰⁻⁹ dilution of the stock inoculum. Based on these data, the infectivity titres for the Chachoengsao/1998 and Ratchaburi/2006 inocula were calculated to be 10¹¹.₅ and 10⁹.₅ LD₅₀/ml, respectively. Quantitative real-time RT-PCR analysis of the inocula indicated that the corresponding viral genetic loads were 1.5×10¹¹ and 3.8×10¹⁰ RNA copies/ml, respectively.

Virus neutralisation assays

Virus neutralisation assays were conducted in SPF *P. vannamei* shrimp using the polyclonal antisera to recombinant [His]₆-tagged gp116 and gp64. For each assay, 10-fold serial dilutions of the stock inoculum were prepared and selected dilutions of virus (10⁻⁸ and 10⁻¹⁰ for Chachoengsao/1998; 10⁻⁶, 10⁻⁷ and 10⁻⁸ for Ratchaburi/2006) were incubated with PBS or a 1/50 dilution of serum. The virus dilutions were selected to approach the pre-determined LD₅₀ for each inoculum. As shown in Fig. 3, anti-gp116 serum effectively neutralised the infectivity of the Chachoengsao/1998 reference isolate ~ 100-fold, with complete neutralisation occurring at an infection dose of 31.6 LD₅₀ and partial (~50%) neutralisation at a dose of 316.2 LD₅₀. Anti-gp64 serum failed to neutralise infectivity and the potency of neutralisation by anti-gp116 serum was not enhanced by co-treatment with anti-gp64 serum, confirming that gp116 is the receptor-binding protein in vivo. However, there was no evidence of neutralisation of the Ratchaburi/2006 isolate by either antiserum, even when applied to a minimal lethal dose of the inoculum (3.126 LD₅₀). In a separate experiment, a 10⁻⁷ dilution (31.62 LD₅₀) of the Ratchaburi/2006 stock inoculum was incubated with gp116, gp64, and mixed gp116 and gp64 antisera at dilutions of 1/25, 1/50 and 1/100. No virus neutralisation was observed (data not shown).

Nucleotide sequence analysis

Genomic RNA isolated from a purified preparation of Ratchaburi/2006 was amplified by RT-PCR using various primers and overlapping regions extending from the ORF2 gene to the 3′-polyA tail were cloned and sequenced. The nucleotide sequence of this ~6.0 kb region was >99.9% identical to that of the Chachoengsao/1998 reference isolate, confirming the identity of the Ratchaburi/2006 isolate as YHV genotype 1. However, the sequence of the Ratchaburi/2006 isolate contained a 162 nt deletion in ORF3 and a single nucleotide deletion in the 3′-UTR. The resulting 54 amino acid deletion in pp3 occurred in the region corresponding to the N-terminal domain of gp116, extending from a site 10 amino acids downstream of the signal peptidase cleavage site and encompassing six conserved cysteine residues and two predicted N-glycosylation sites in the Chachoengsao/1998 isolate (Fig. 4).

![Fig. 2. Infectivity titrations in SPF *P. vannamei* shrimp of stock inoculums of YHV isolates Chachoengsao/1998 and Ratchaburi/2006. Groups of 15 shrimp were injected with 10-fold serial dilutions of virus in 0.1% BSA in PBS and observed daily for signs of disease. Moribund and dead shrimp were collected from the tanks and recorded as mortalities.](image-url)
The ORF3 region containing the deletion was also examined for 25 isolates comprising representatives of the six known genotypes in the YHV complex. DNA products amplified by RT-PCR were sequenced and ClustalX was used to align amino acid sequences deduced for Chachoengsao/1998 (THA-98-Ref), Ratchaburi/2006 (THA-06-D1) and five other isolates of YHV (genotype 1), eight isolates of GAV (genotype 2), six isolates of genotype 3, two isolates of genotype 4, and one isolate each of genotype 5 and genotype 6 (Fig. 4). A high level of sequence identity was evident among isolates of genotypes 2, 3, 4, 5 and 6. These isolates also shared common structural features including the cluster of six conserved cysteine residues and three potential N-linked glycosylation sites, one of which was absent only from genotype 4. However, the YHV isolates varied significantly from other genotypes and clustered into two distinct sequence patterns corresponding to the Chachoengsao/1998 and Ratchaburi/2006 sequences. Examination of the previously reported genotype assignments based on a 641 nt amplicon in ORF1b (Wijegoonawardane et al., 2008a) indicated that the two other isolates sharing the 162 nt deletion in ORF3 corresponded to YHV genotype 1b. The analysis of these sequences confirms the existence of two YHV sub-types (genotypes 1a and 1b), isolates of which have been obtained only from Thai shrimp displaying gross signs of YHD.

**Discussion**

Yellow head virus contains two envelope glycoproteins (gp116 and gp64) generated by post-translational cleavage of the large pp3 polyprotein encoded in ORF3 (Jitrapakdee et al., 2003). Cleavage occurs at two signal peptidase type 1 sites located immediately downstream of transmembrane domains 2 and 5 of the six predicted in pp3 (Cowley and Walker, 2002; Jitrapakdee et al., 2003). Assuming cleavage occurs with equal efficiency at each site, stoichiometrically equivalent amounts of gp116 and gp64 (and an N-terminal triple-membrane spanning fragment of unknown function) would arise from the pp3 precursor polyprotein. Indeed, in the Chachoengsao/1998 reference strain of YHV, gp116 and gp64 occur in virions in roughly equimolar proportions (Jitrapakdee et al., 2003). However, analysis of the Ratchaburi/2006 isolate from farmed *P. vannamei* shrimp with typical signs of yellow head disease indicated that its gp116 is smaller than in the Chachoengsao/1998 reference isolate, and exists in virions at a relatively lower molar ratio to gp64. Sequence analysis of the structural gene region of the Ratchaburi/2006 isolate identified a 54 amino acid deletion in the N-terminal domain of gp116 that eliminated two of seven potential N-linked glycosylation sites and six of the 24 highly conserved cysteine residues that are likely to form disulphide bridges to stabilise the secondary structure of the
ectodomain (Jitrapakdee et al., 2003). Perhaps not surprisingly, the sequence deletion in gp116 was shown to cause a structural modification, greatly reducing its reactivity in immunoblots and rendering the Ratchaburi/2006 isolate resistant to neutralisation in vivo by polyclonal anti-gp116 serum raised to the Chachoengsao/1998 reference strain.

Despite the structural deformation in gp116Δ and the relatively lower number of molecules presented at the surface of virions, the Ratchaburi/2006 isolate remained infectious and virulent for shrimp, both in the field and in laboratory challenge experiments. Comparative titrations of the two isolates did indicate that the LD50 of a standard inoculum of the Ratchaburi/2006 isolate was approximately 100-fold lower than that of the reference strain. By qRT-PCR, the viral genetic load of the Chachoengsao/1998 strain was similar to the LD50 (1.5×1011 copies/ml) and the Ratchaburi/2006 isolate was only ∼5-fold lower. This appears to indicate that there is some correlation between infectivity and the level of incorporation of gp116 or gp116Δ into virions. However, the significance of this is not yet clear as, in the absence of a reliable in vitro assay (due to the lack of suitable cell lines), it has not been possible to assess accurately the relative infectivity or relative virulence of the two inocula. Nevertheless, the infectivity titre of the Ratchaburi/2006 isolate is very high (109.5 LD50/ml) and the virus is clearly capable of efficient infection.

The precise nature of the structural deformation in the Ratchaburi/2006 gp116Δ is not yet clear. Although some refolding may have occurred during transfer following SDS-PAGE, the absence of reactivity in immunoblots of monoclonal antibody V3-2B and the very poor reactivity of anti-gp116 polyclonal rabbit serum suggests that the deleted 54 amino acid region of gp116 is the major site of immunodominant linear epitopes. However, this region does not appear to contain the receptor-binding site as the Ratchaburi/2006 isolate retains a high level of infectivity and virulence. The failure of anti-gp116 polyclonal serum to neutralise the infectivity of the Ratchaburi/2006 isolate indicates that the receptor-binding site, although still functional, is no longer concealed by binding of the neutralising antibody. This could occur if the N-terminal region of gp116 forms an autonomous domain or elongated loop at the surface of the protein to which neutralising antibodies bind, blocking access to a nearby receptor-binding site. Secondary structures predicted using the PHD algorithm (http://www.embl-heidelberg.de/predictprotein/predictprotein.html) suggest that the region of gp116 corresponding to the 54 amino acid deletion can indeed form a loop containing helical domains stabilised by three disulphide bridges. According to this model, the absence of this N-terminal loop domain in gp116Δ would eliminate the neutralisation site but preserve the receptor-binding site.

Alternatively, it could be argued that the Ratchaburi/2006 isolate remains infectious by adopting an alternative gp116-independent pathway of cell attachment and entry. Sequence analysis indicated that the second envelope glycoprotein (gp64) remains intact in the Ratchaburi/2006 isolate and it appears to be incorporated efficiently into virions. However, anti-gp64 polyclonal rabbit serum failed to neutralise either the Chachoengsao/1998 or the Ratchaburi/2006 isolate, indicating that gp64 is not targeted by neutralising antibody and is not an alternative mediator of cell attachment. This is consistent with previous observations in vitro that neutralising antibody targets...
only gp116 (Assavalapsakul et al., 2005). Aaskov et al. (2006) have reported evidence that defective envelope glycoprotein (E) is carried in dengue virus populations circulating in mosquitoes and humans, most likely by complementation by viable strains. However, in purified virions of the Ratchaburi/2006 isolate, there was no evidence of a second larger glycoprotein band that may have been contributed by a complementing virus and, even if present at low levels, the infectivity of virions containing full-length gp116 would have been neutralised by the anti-gp116 rabbit serum. There was also no evidence of a second longer DNA product when the ORF3 region containing the deletion was amplified by RT-PCR, again suggesting the absence of a complementing virus. It appears, therefore, that the deformation of gp116Δ in the Ratchaburi/2006 isolate does not completely eliminate the intrinsic receptor-binding function, although it appears to have affected the efficiency of its incorporation into virions.

Longyant et al. (2006) have reported that the tissues of several palaemonid shrimp species experimentally infected with YHV displayed strong reactivity to gp64 antibody but weak and inconsistent reactions to gp116 antibody, and have interpreted this finding as selective inhibition of gp116 expression. Chailisuthangkura et al. (2008) have also recently reported preferential suppression of gp116 expression in P. vannamei and Palaemon serrifer shrimp that survived experimental challenge with YHV. As gp116 is generated by processing of the ORF3 polyprotein (pp3), which also generates the downstream gp64 fragment, poor or inconsistent gp116 immuno-reactivity in the tissues of infected shrimp was explained by selective degradation following expression. Preferential suppression of glycoprotein expression has been described previously as a host-directed mechanism for maintaining persistence of vesicular stomatitis virus (VSV) infection in arthropod cells (Wyers et al., 1980) and there is evidence that interferon can preferentially suppress the incorporation of VSV G protein into virions by disrupting the maturation process in mammalian cells (Maheshwari et al., 1980; Jay et al., 1983). However, although other mechanisms could be invoked, the apparent preferential suppression of YHV gp116 expression reported in shrimp is most likely the consequence of natural and experimental infections with YHV strains possessing a variant gp116Δ similar to that detected in Ratchaburi/2006. The detection of identical 54 amino acid deletions in two other YHV stains from diseased P. monodon in Thailand in 2002 and 2003 (Fig. 4) indicates that the variant gp116Δ occurs commonly in cultured shrimp and may well have been present in the virus preparations used by previous authors in experimental studies.

It has been reported recently that YHV is one of at least six genotypes in the yellow head complex that occur commonly in farmed P. monodon shrimp in the Indo-Pacific region (Wijegoonawardane et al., 2008a, 2008b). Analysis of a 671 nt amplicon in the ORF1b (replicase) gene has indicated that YHV isolates form two subtypes with each of three other genotype 1b isolates (genotypes 1a and 1b), each of which has been isolated from shrimp (replicase) gene has indicated that YHV isolates form two subtypes with each of three other genotype 1b isolates (genotypes 1a and 1b), each of which has been isolated from shrimp.

Materials and methods

Source of viruses

The Chaohongsa/1998 reference strain of YHV (THA-98-Ref) was isolated from P. monodon shrimp displaying typical signs of yellow head disease collected from a pond in Chaohongsa Province, East-Central Thailand, in July 1998 (Sittidiolokratna et al., 2002). The Ratchaburi/2006 YHV strain (THA-06-D1) was isolated from 24-day-old P. vannamei shrimp collected from a yellow head disease outbreak pond at Bang-Pae in Ratchaburi Province, West-Central Thailand, in February 2006. The origins of other isolates, representing each of the six known genotypes in the yellow head complex, have been described elsewhere (Wijegoonawardane et al., 2008a).

Virus purification

YHV was purified from clarified hemolymph collected from 100–200 experimentally infected P. monodon shrimp by velocity sedimentation in 20%–40% Urografin density gradients as described previously (Wongteerasupaya et al., 1995a, 1997). Gradient fractions containing virions were collected and diluted, and the virus pellet obtained following ultracentrifugation was resuspended in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.4) and stored in aliquots at –80 °C.

Recombinant baculoviruses

Recombinant baculoviruses expressing the ectodomains of YHV envelope glycoproteins gp116 and gp64 of the Chaohongsa/1998 strain were prepared using two different baculovirus expression systems. For gp116, the ORF3 region spanning T225–L1010 in pp3 was amplified from YHV genomic RNA by using the SuperScript one-step RT-PCR system (Invitrogen) in a 25 μl reaction containing ~100 ng RNA, 8 U RNasin (Promega) and 0.4 μM of each primer (Table 1). The forward primer (SS9) included a SalI site and the reverse primer (SA8) included a NotI site and the termination codon. DNA was amplified in a Perkin Elmer 2400 thermal cycler using the cycling conditions 50 °C/30 min, 94 °C/2 min, 35 cycles of 94 °C/30 s, 68 °C/2.5 min and then 72 °C/10 min. The DNA product was gel-purified using a QiAgel Quick Extraction kit (QIAGEN) and cloned into pGEM-T Easy vector (Promega). Inserts were verified by direct sequencing of the recombinant plasmids. The insert in pGEM-T-T13 was excised using SalI and NotI and ligated into the transfer vector pFastBacExporTHIS (containing the Lucilia cuprina peritrophin-95 signal peptide sequence followed by an N-terminal [His]6-tag; Casu et al., 1997) to derive the plasmid pFastBacExporTHIS-T13-13. The recombinant plasmid sequence was verified before transformation of E. coli MAX Efficiency DH10Bac. Recombinant bacmid DNA isolated from white colonies was amplified by PCR using M13 forward and reverse primers to verify successful insert transposition into the bacmid. Sf9 insect cells were transfected with bacmid DNA using Cellfectin reagent (Invitrogen) and recombinant baculovirus recovered from the cell cultures was amplified by additional passages and expressed gp116 protein was detected by SDS–PAGE and immunoblotting.

For gp64, the ORF3 region spanning the ectodomain (L1328 to D1526 in pp3) was amplified from cDNA insert in a pUC18 (Jitrapakdee et al., 2003). The forward primer (Hisgp64F) included an Ncol site providing an initiation codon and reverse primer (Hisgp64R), included a [His]6 coding sequence, a termination codon and an EcoRI site (Table 1). DNA was amplified using the cycling conditions 94 °C/2 min and 30 cycles of 94 °C/1 min, 55 °C/1 min and 72 °C/2 min. The DNA product was cloned into pGEM-T Easy vector and the insert in pGEM-Hisgp64 was verified by sequencing. The pGEM-Hisgp64 insert was then excised using Ncol and EcoRI, and ligated into the baculovirus expression vector pBAC-3 (Novagen, USA). To produce recombinant baculovirus,
Table 1

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Primers for amplification of Ratchaburi/2006 genome sequences

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* Restriction endonuclease sites are underlined; the termination codon (SA8) and hexahistidine tag (Hisgp64R) are indicated in bold face.

pBAC-3 DNA and triple-cut baculovirus DNA (BacVector 1000, Novagen) were co-transfected into S9 cells. Recombinant baculovirus recovered from the cell cultures was amplified by additional passages and expressed gp64 protein was detected by SDS-PAGE and immunoblotting.

Purification of recombinant proteins

Recombinant [His]6-tagged gp116 and gp64 were purified from S9 cell lysates by Ni2+–NTA affinity chromatography (Crowe et al., 1995). The purified proteins were refolded by rapid dilution (1/10) in refolding buffer (20% glycerol in PBS pH 7.4), followed by gentle mixing at room temperature for 1 h. The proteins were then dialysed twice against 1 l PBS for 2 h and concentrated using an Amicon 10 kDa ultrafiltration column (Millipore, USA).

Rabbit immunization and antibody production

Polyclonal rabbit antisera were produced at the Center for Agricultural Biotechnology, Nakhon Pathom, Thailand. Briefly, rabbits were immunized subcutaneously with 1 ml of recombinant protein (1 μg/μl) emulsified in Freund’s complete adjuvant (1:1) followed after 2 weeks by a second 1 ml dose (1 μg/μl) in Freund’s incomplete adjuvant. Blood was collected at 2 week intervals after the second injection and the presence of gp116- and gp64-specific antibodies in the antisera was confirmed by SDS-PAGE and immunoblotting.

Mouse monoclonal antibodies were kindly provided by Dr Paisarn Sinthigornkul, Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok. The production of the antibodies and their specificities for YHV structural proteins [V3-28 (gp116-specific), Y18 (gp64-specific) and Y19 (p20-specific)] have been described in detail elsewhere (Sinthigornkul et al., 2000, 2002).

SDS-PAGE and immunoblotting

YHV virion proteins were separated by SDS-PAGE using 7.5% or 10% discontinuous gels (Laemmli, 1970) and either stained with Coomassie Brilliant Blue R-250 or transferred onto PVDF membranes for 2 h at room temperature using a semi-dry blotting apparatus (Hoefer, USA). Membranes were incubated overnight in PBST (0.05% Tween-20 in PBS) containing 5% skim milk powder and 3% BSA (blocking buffer) and then incubated with primary antibody in this blocking buffer for 1 h at room temperature with gentle rocking. Monoclonal antibodies were used at 2 × 10−4 dilution and polyclonal rabbit antisera were used at 10−5 dilution to avoid non-specific reactions. Membranes were washed three times for 10 min in PBST and then incubated for 1 h at room temperature with 1/5000 horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit IgG in blocking buffer, and washed three times in PBST. ECL substrate (Amersham Pharmacia) was then added for 1 min and immuno-reactive proteins were detected using ECL-film.

Experimental infection of shrimp

Experimental infections were conducted in specific pathogen-free (SPF) P. vannamei shrimp that had been reared in biosecure polyethylene-lined ponds at Ladyai, Thailand. Shrimp (1.5–5.0 g) were maintained at 27 °C–31 °C in 50 L rectangular tanks (12–20 shrimp/tank) containing 35 L of 15 ppt seawater. Shrimp were acclimated for 3 days on arrival at the facility and reared for 14 days prior to experimental use. Water was exchanged every 3 days and shrimp were fed 4 times/day. Prior to use, batches of shrimp were screened to confirm the absence of pre-existing viral infections by using IQ2000 PCR kits for detection of WSSV, YHV/GAV, TSV and IHHNV (Farming Intelligene Technology Corporation, Taiwan).

Stock preparations of viruses for experimental infections were prepared by passage in healthy adult P. monodon of filtered (0.4 μm), emulsified cephalothorax tissue extracts from diseased shrimp. Hemolymph collected from the shrimp was pooled and stored at −80 °C in aliquots. Serial 10-fold dilutions of hemolymph were prepared in sterile PBS pH 7.4 containing 0.1% BSA. Infectivity titrations were conducted using triplicate groups of 12 P. vannamei shrimp per dilution. Shrimp (2–5 g) were injected with 100 μl each inoculum into the third abdominal segment and observed daily. Moribund and dead shrimp were removed from tanks and scored as mortalities.

Virus neutralisation assays were conducted using stock preparations of virus at selected dilutions in sterile PBS (pH 7.4) containing
0.1% BSA. The diluted virus was mixed with dilutions of pre-bleed rabbit serum, anti-gp116 rabbit serum or anti-gp64 rabbit serum, and incubated at 30 °C for 90 min. Triplicate groups of 15 or 20 P. vannamei shrimp (2–5 g) were injected as above and observed daily. Moribund and dead shrimp were removed from tanks and scored as mortalities.

Quantitative real-time RT-PCR (qRT-PCR)

Quantitative real-time RT-PCR was conducted on RNA extracted from the viral inocula by using the SYBR Green method and the YHV forward (135 Fwd) and reverse (135 Rev) primers described by Wongteerasupaya et al. (1995b). cDNA was synthesized in a 25 μl reaction in Superscript RT buffer (Invitrogen) containing 48 ng RNA, 500 nM dNTPs, 500 μM primer 135 Rev, 4 mM dithiothreitol and 200 U Superscript II reverse transcriptase. The reaction was incubated at 25 °C/5 min, 52 °C/30 min following by 70 °C/15 min. PCR was conducted in a 25 μl reaction containing SYBR PCR mix (QIAGEN), 1 μl cDNA and 300 nM YHV primers. The thermal cycling conditions were 95 °C/15 min, and 40 cycles of 95 °C/15 s, 52 °C/30 s, and 72 °C/45 s. A ten-fold dilution series of YHV plasmid DNA was used as a quantification standard.

RNA extraction, PCR amplification and sequence analysis

RNA was extracted from purified virions of the Ratchaburi/2006 YHV isolate using the RNAeasy Mini Kit (QIAGEN) according to manufacturer’s instructions. PCR primers were designed to amplify overlapping segments from a site 30 nt downstream of the ORF2 initiation codon through to the 3′-polyA tail (Table 1). RT-PCR was conducted using the SuperScript III One-Step RT-PCR System with Platinum® Tag DNA polymerase (Invitrogen). Reactions were performed according to the manufacturer’s instructions using kit reagents plus 200 ng of genomic RNA, 0.2 μM primers and a final concentration of 0.1 mM MgSO4. DNA was amplified in a Perkin Elmer 2400 thermal cyclers using the cycling conditions 50 °C/30 min, 94 °C 2 min followed by 35 cycles of 94 °C/30 s, 55 °C/30 s, 72 °C/1 min, and a final extension of 72 °C/10 min. PCR products were cloned into pGEM-T Easy vector and the sequence was determined in each direction using T7 and SP6 primers. The contigs were aligned using ClustalX and the consensus was determined using the Staden package. The consensus sequence was further analyzed using phylogenetic analyses with the MEGA software (Tamura et al., 2011). The neighbor joining tree was constructed and the nucleotide substitution matrix with Kimura’s 2-parameter model of nucleotide substitution. Bootstrap values were calculated with 100 iterations.

Nucleotide sequence deposition

Nucleotide sequences reported in this paper have been deposited in GenBank under accession number FJ194948.9.

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