Virology 384 (2009) 192–200

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

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

Nusra Sittidilokratna [a,b,1], Charoonroj Chotwiwatthanakun [b], Priyanjalie KM Wijegoonawardane [c,2], Sasimanas Unajak [b], Anutara Boonnad [d], Watcharakorn Wangnai [d], Sarawut Jitrapakdee [f], Jeff A Cowley [c], Peter J Walker [c,e,*]

[a] National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Phathumthani 12120, Thailand
[b] Center of Excellence for Shrimp Molecular Biology and Biotechnology, Faculty of Science, Mahidol University, Rama VI Road, Phayathai, Bangkok 10400, Thailand
[c] CSIRO Livestock Industries, Queensland Bioscience Precinct, 306 Carmody Road, St Lucia, Queensland 4067, Australia
[d] Shrimp Culture Research Center, Chaoren Pokphand Foods PCL, Rama II Road, Samut Sakorn, Thailand
[e] CSIRO Livestock Industries, Australian Animal Health Laboratory, 5 Portarlington Road, Geelong, Victoria 3220, Australia
[f] Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok 10400, Thailand

ARTICLE INFO

Article history:
Received 11 September 2008
Returned to author for revision
17 October 2008
Accepted 28 October 2008
Available online 2 December 2008

Keywords:
Yellow head virus
Nidovirus
Neutralisation
Penaeus vannamei
Shrimp
Glycoprotein

ABSTRACT

Yellow head virus (YHV) is a highly virulent pathogen of penaeid shrimp. An isolate obtained from *Penaeus vannamei* during a yellow head disease outbreak in February 2006 in Ratchaburi Province, Thailand was purified following passage in experimentally infected shrimp. SDS-PAGE of purified virions indicated that envelope glycoprotein gp116 in the Ratchaburi/2006 isolate was smaller and relatively less abundant than in the Chachoengsao/1998 YHV reference strain. The variant gp116 reacted poorly in immunoblots with a gp116 mouse monoclonal antibody and a rabbit anti-serum to a baculovirus-expressed, C-terminally truncated, $[His]_6$-tagged gp116 that reacted strongly with gp116 of the homologous Chachoengsao/1998 strain. The anti-gp116 polyclonal serum also failed to neutralise the infectivity of the Ratchaburi/2006 isolate in *in-vivo* assays conducted in *P. vannamei*, but effectively neutralised the infectivity of the reference strain. Sequence analysis of the ~6.0 kb structural protein gene region and 3′UTR of the Ratchaburi/2006 isolate indicated >99.9% overall nucleotide identity with the Chachoengsao/1998 strain. However, in Ratchaburi/2006 a deletion in ORF3, corresponding to 54 amino acids near the N-terminal signal peptidase cleavage site of gp116, resulted in the loss of six conserved cysteine residues and two predicted N-glycosylation sites. Analysis of this ORF3 region in 25 viruses representing each of the six genotypes in the yellow head complex identified this modified form of gp116 in two other virulent YHV isolates classified as genotype 1b. The data indicate that, although the deletion causes a significant structural deformation of gp116 which reduces its incorporation into virions and eliminates the major neutralisation sites, the virus remains highly infectious, virulent and fit for survival.

Introduction

Yellow head virus (YHV) is a major pathogen of the black tiger 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, exonuclease, uridylate-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

* Corresponding author. CSIRO Livestock Industries, Australian Animal Health Laboratory, 5 Portarlington Road, Geelong, Victoria 3220, Australia. Fax: +61 3 5227 5555.
E-mail address: Peter.Walker@csiro.au (P.J. Walker).
[1] CSIRO Livestock Industries, Australian Animal Health Laboratory, 5 Portarlington Road, Geelong, Victoria 3220, Australia.
[2] National Aquatic Resources Research and Development Agency, Crow Island, Mattakkuliya, Colombo 15, Sri Lanka.

0042-6822/$ – see front matter.
doi:10.1016/j.virol.2008.10.042

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 (Longyant 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 antisera 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

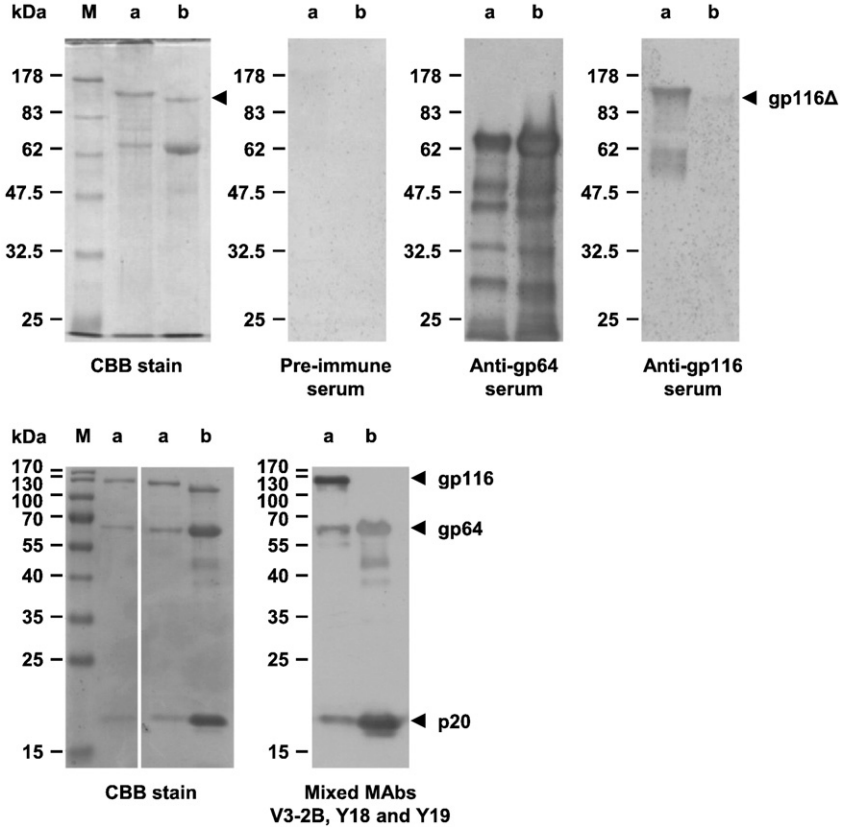

Fig. 1. SDS-PAGE and immunoblot analysis of purified virus preparations of YHV isolates (a) Cachoengsao/1998 and (b) Ratchaburi/2006. Gels were either fixed and stained with Coomassie brilliant blue R-250 or transferred to PDVF membranes for immunoblotting with pre-immune rabbit serum, anti-gp64 serum, 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).

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 LD_{50} 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 Rarchaburi/2006, the minimum lethal dose, which also resulted in 100% cumulative mortality at day 6, was 100-fold higher (10^{-8} dilution of the stock inoculum). Based on these data, the infectivity titres for the Chachoengsao/1998 and Ratchaburi/2006 inocula were calculated to be $10^{11.5}$ and $10^{9.5}$ LD_{50}/ml, respectively. Quantitative real-time RT-PCR analysis of the inocula indicated that the corresponding viral genetic loads were 1.5×10^{11} and 3.8×10^{10} 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^{-9} 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 LD_{50} 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 LD_{50} and partial (~50%) neutralisation at a dose of 316.2 LD_{50}. 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 LD_{50}). In a separate experiment, a 10^{-7} dilution (31.62 LD_{50}) 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).

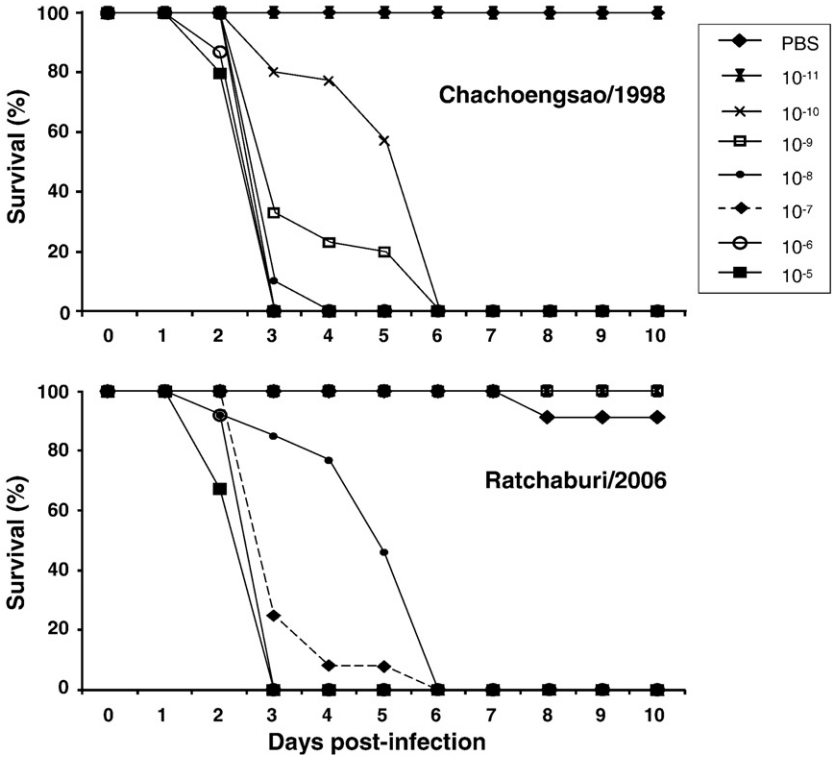

Fig. 2. Infectivity titrations in SPF *P. vannamei* shrimp of stock inoculums of YHV isolates Chachoengsao/1998 and Ratchaburi/2006. Groups of 15 shrimp were injected with 10-fold serial dilutions of virus in 0.1% BSA in PBS and observed daily for signs of disease. Moribund and dead shrimp were collected from the tanks and recorded as mortalities.

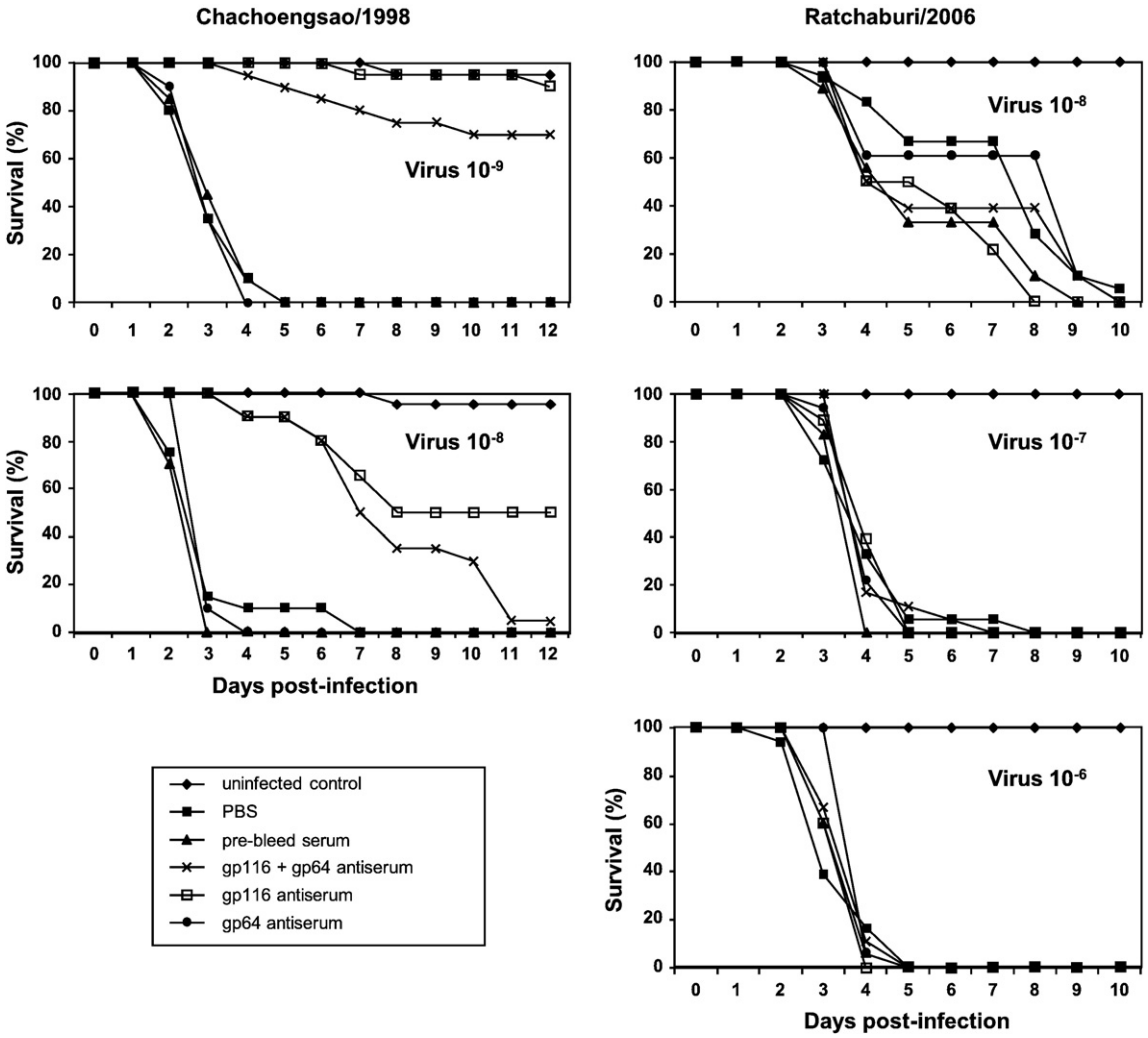

Fig. 3. Virus neutralisation assays of YHV isolates Chachoengsao/1998 and Ratchaburi/2006. Triplicate groups of 15 shrimp were injected with 10-fold serial dilutions of virus in 0.1% BSA in PBS alone or containing 1/50 dilutions of (i) pre-bleed rabbit serum; (ii) anti-gp116 rabbit antiserum; (iii) anti-gp64 rabbit serum; or (iv) a mixture of anti-gp116 and gp64 rabbit sera. Control shrimp were untreated. The shrimp were observed daily for signs of disease and moribund and dead shrimp were collected and recorded as mortalities. Survival curves are illustrated for virus inoculum dilutions of 10^{-8} (316.2 LD_{50}) and 10^{-9} (31.62 LD_{50}) for the Chachoengsao/1998 isolate and 10^{-6} (316.2 LD_{50}), 10^{-7} (31.62 LD_{50}) and 10^{-8} (3.162 LD_{50}) for the Ratchaburi/2006 isolate.

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

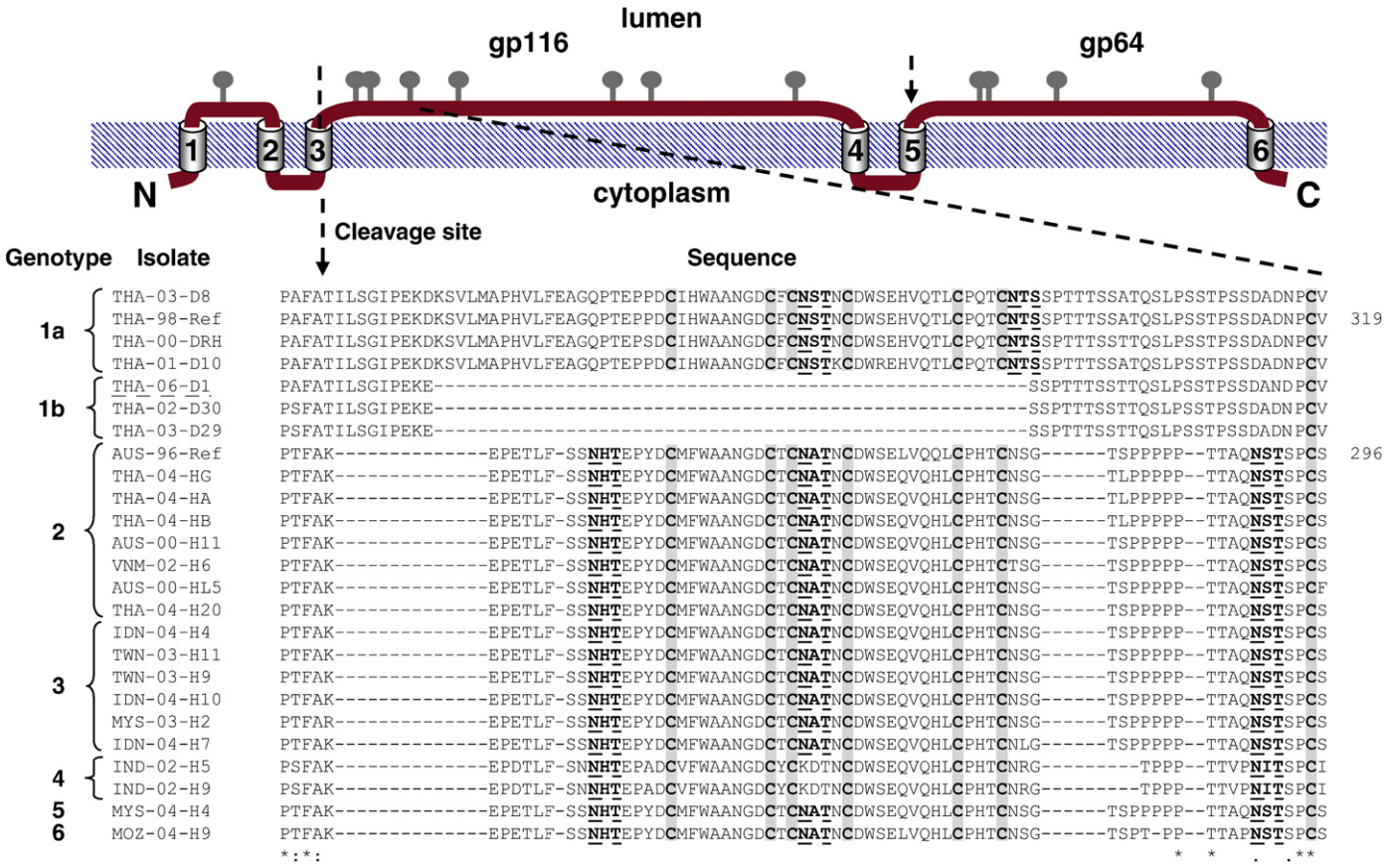

Fig. 4. A ClustalX multiple alignment of amino acid sequences spanning the N-terminal gp116 region of ORF3 of viruses in the YHV complex representing each of the major genotypes. The Chachoengsao/1998 reference isolate is included (THA-98-Ref). The Ratchaburi/2006 isolate (THA-06-D1) is indicated in bold and underlined. The assignment of other genotypes is according to the phylogenetic analysis of sequences in ORF1b (Wijegoonawardane et al., 2008a). The site of proteolytic cleavage of the YHV pp3 polyprotein is indicated. Absolutely conserved (*) and similar (: or .) amino acids are indicated according to the similarity groups defined in ClustalX. Conserved cysteine residues are indicated in bold face with shadowing. Potential N-linked glycosylation sites are indicated in bold face and underlined in the alignment and denoted () in the illustration. Six predicted transmembrane-spanning domains (TM1–TM6) are numbered. Amino acid positions from the start of pp3 are indicated for the two reference isolates (THA-98-Ref and AUS-96-Ref).

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 LD_{50} 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 LD_{50} (1.5×10^{11} 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 ($10^{9.5}$ LD_{50}/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. Chaivisuthangkura 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 immunoreactivity 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 (genotypes 1a and 1b), each of which has been isolated from shrimp with typical signs of yellow head disease in Thailand. The data presented here indicates that the Ratchaburi/2006 isolate shares the deletion in gp116 with each of three other genotype 1b isolates detected previously among 16 YHV isolates from *P. monodon* collected from disease outbreaks in Thailand and Taiwan from 1998 to 2003. The earliest genotype 1b isolate appeared in diseased *P. monodon* juveniles in Central Thailand in 2003 (Wijegoonawardane et al., 2008a). Ratchaburi/2006 is the first isolate of this subtype to be recovered from *P. vannamei*, which is endemic to the Americas and recently introduced to Thailand as an alternative culture species. It appears, therefore, that the genotype1b viruses represent an efficiently replicating variant lineage that is actively spreading among these shrimp populations. Work is continuing to further characterise the structural deformation and how gp116Δ retains cell receptor-binding capability and the maintenance of a virulent phenotype.

Materials and methods

Source of viruses

The Chachoengsao/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 Chachoengsao Province, East-Central Thailand, in July 1998 (Sittidilokratna 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 Chachoengsao/1998 strain were prepared using two different baculovirus expression systems. For gp116, the ORF3 region spanning T^{229}–I^{1010} 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 *Sal*I site and the reverse primer (SA8) included a *Not*I site and a 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 QIAquick Gel 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 *Sal*I and *Not*I 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 (L^{1128} to D^{1626} in pp3) was amplified from cDNA insert in a pUC18 (Jitrapakdee et al., 2003). The forward primer (Hisgp64F) included an *Nco*I site providing an initiation codon and reverse primer (Hisgp64R), included a $[His]_6$ coding sequence, a termination codon and an *Eco*RI 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 *Nco*I and *Eco*RI, and ligated into the baculovirus expression vector pBAC-3 (Novagen, USA). To produce recombinant baculovirus,

Table 1
PCR primer sequences

Primer	Sequence (5′-3′)[a]	
Primers for cloning of glycoprotein genes in baculovirus expression vectors		*ORF3 region*
SS9	CCTGCTGTCGACACGATTCTAAGTGGAATTCCTGAA	T^{229}–I^{1010}
SA8	ATAGGAGAAGGCGGCCGC**CTA**GATTTGATCTTTGAGAATGGA	
Hisgp64F	GCCATGGGCCTCGCTCCACGACAGGCACGT	L^{1128}–D^{1626}
Hisgp64R	GAATTCTCA**ATGATGATGATGATGATG**GCCGCCATCCCATGTCTTGCCGCCGAATGCGAAAGGAGT	
Primers for amplification of Ratchaburi/2006 genome sequences		*Size (bp)*
2S041	ATGCCTCGTCGTCGCCTAC	900
2A044	CAAGGAAGTATCGTTGGACGG	
2S042	GCACCAGGCTCGCATATCAT	880
YA11	GTACATACAGCCAGGGACTG	
2S043	TCTGACATTGTATCTTTATACCG	860
2A045	AGTAGATGAAGAAACGCTTAGG	
YS2	GGAAGAGTATCATGACGGAG	880
YA5	GAACGCCAAGAGAGTATGGT	
YS8	TTCTGGCTACATCTTCAGAGA	680
YA2	GCGTTGAGGCACGGTATAAC	
YS3	GGTTATACCGTGCCTCAACG	860
2A046	TAATTGTCTTGAAGTCTGCGG	
2S044	ATGCGCATCCATCACAACAG	900
2A047	ATGTCTTGCCGCCGAATGC	
2S045	GGTGCTGCAGGTAATGATGG	860
2A048	TTTTTTTTTTTTTTTTCATATCACCG	
YF001	TTGCCCATGATAGACATAAGC	1432
YR001	CGAACAACACTACCAGCTGTT	

[a] 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 Sf9 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 Sf9 cell lysates by Ni^{+2}-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 ultra MW exclusion 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 Sithigorngul, Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok. The production of the antibodies and their specificities for YHV structural proteins [V3-2B (gp116-specific), Y18 (gp64-specific) and Y19 (p20-specific)] have been described in detail elsewhere (Sithigorngul 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 virus 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 pM primer 135 Rev, 4 mM dithiothreitol and 200 U Superscript II reverse transcriptase. The reaction was incubated at 25 °C/5 min, 52 °C/60 min followed 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® *Taq* 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 $MgSO_4$. DNA was amplified in a Perkin Elmer 2400 thermal cycler 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 entire sequence was constructed manually. The deletion in ORF3 was confirmed by an additional RT-PCR amplification of genomic RNA using primers YF001 and YR001 (Table 1). The ~1.4 kb DNA product was cloned into pGEM-T Easy and sequenced in both directions using T7, SP6 and internal primers.

The procedures for RNA extraction, RT-PCR amplification and sequence analysis of a ~1250 nt region of ORF3 for other isolates representing the six genotypes in the YHV complex are described elsewhere (Wijegoonawardane et al., submitted).

Nucleotide sequence deposition

Nucleotide sequences reported in this paper have been deposited in GenBank under accession number FJ194949.

Acknowledgments

The authors wish to thank Dr Rosanne Casu, CSIRO Plant Industry, Brisbane, for providing baculovirus expression vector pFastBacExporTHIS, Dr Kallaya Sritunyalucksana and Ms Sirintip Dangtip of Centex Shrimp Bangkok for conducting qPCR assays, and Dr Sarawut Jitrapakdee of Mahidol University, Bangkok, for advice on the use of the baculovirus expression system. The authors also wish to acknowledge Mr Supol Prawai and Mr Tawee Ong-na-non of the Shrimp Culture Research Center, Chaoren Pokphand Foods Public Company Limited for assistance with experimental infections of shrimp and Dr Yves Gaudin, CNRS, Gif-sur-Yvette, France, for advice on glycoprotein structure.

References

Aaskov, J., Buzacott, K., Thu, H.M., Lowry, K., Holmes, E.C., 2006. Long-term transmission of defective RNA viruses in humans and *Aedes* mosquitoes. Science 311, 236–238.

Assavalapsakul, W., Tirasophon, W., Panyim, S., 2005. Antiserum to the gp116 glycoprotein of yellow head virus neutralizes infectivity in primary lymphoid organ cells of *Penaeus monodon*. Dis. Aquat. Org. 63, 85–88.

Casu, R., Eismann, C., Pearson, R., Riding, G., East, I., Donaldson, A., Cadogan, L., Tellam, R., 1997. Antibody-mediated inhibition of the growth of larvae from an insect causing cutaneous myiasis in a mammalian host. Proc. Natl. Acad. Sci. U. S. A. 94, 8939–8944.

Chaivisuthangkura, P., Tejangjura, T., Rukpratanporn, S., Longyant, S., Sithigorngul, W., Sithigorngul, P., 2008. Preferential suppression of yellow head virus (YHV) envelope protein gp116 in shrimp that survive challenge with YHV. Dis. Aquat. Org. 79, 1–8.

Chantanachookin, C., Boonyaratpalin, S., Kasornchandra, J., Direkbusaratana, S., Ekpanithanpong, U., Supamataya, K., Sriurairatana, S., Flegel, T.W., 1993. Histology and untrastructure reveal a new granulosis-like virus in *Penaeus monodon* affected by yellow-head disease. Dis. Aquat. Org. 17, 145–157.

Cowley, J.A., Walker, P.J., 2002. The complete sequence of gill-associated virus of *Penaeus monodon* prawns indicates a gene organisation unique among nidoviruses. Arch. Virol. 147, 1977–1987.

Cowley, J.A., Dimmock, C.M., Spann, K.M., Walker, P.J., 2000. Gill-associated virus *Penaeus monodon* prawns: an invertebrate nidovirus with ORF1a and ORF1b gene related arteri- and coronaviruses. J. Gen. Virol. 81, 1473–1484.

Cowley, J.A., Cadogan, L.C., Spann, K.M., Sittidilokratna, N., Walker, P.J., 2004. The gene encoding the nucleocapsid protein of gill-associated nidovirus of *Penaeus monodon* prawns is located upstream of the glycoprotein gene. J. Virol. 78, 8935–8941.

Crowe, J., Masone, B.S., Ribbe, J., 1995. One-step purification of recombinant proteins with the 6xHis tag and Ni^{2+}-NTA resin. Mol. Biotechnol. 4, 247–258.

Jay, F.T., Dawood, M.R., Friedman, R.M., 1983. Interferon induces production of membrane protein-deficient and infectivity-defective vesicular stomatitis virus through interference in the virion assembly process. J. Gen. Virol. 64, 707–712.

Jitrapakdee, S., Unajak, S., Sittidilokratna, N., Hodgson, R.A.J., Cowley, J.A., Walker, P.J., Panyim, S., Boonsaeng, V., 2003. Identification and analysis of gp116 and gp64 structural glycoproteins of yellow head nidovirus of *Penaeus monodon* shrimp. J. Gen. Virol. 84, 863–873.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Limsuwan, C., 1991. Handbook for Cultivation of Black Tiger Prawns. Tamsetakit Co. Ltd., Bangkok. (in Thai).

Longyant, S., Sattaman, S., Chaivisuthangkura, P., Rukpratanporn, S., Sithigorngul, W., Sithigorngul, P., 2006. Experimental infection of some penaeid shrimps and crabs by yellow head virus (YHV). Aquaculture 257, 83–91.

Maheshwari, R.K., Jay, F.T., Friedman, R.M., 1980. Selective inhibition of glycoprotein and membrane protein of vesicular stomatitis virus from interferon-treated cells. Science 207, 540–541.

Nadala, E.C.B., Tapay, L.M., Loh, P.C., 1997. Yellow-head virus: a rhabdovirus-like pathogen of penaeid shrimp. Dis. Aquat. Org. 31, 141–146.

Sithigorngul, P., Chauychuwong, P., Sithigorngul, W., Longyant, S., Chaivisuthangkura, P., Menasveta, P., 2000. Development of a monoclonal antibody specific to yellow head virus (YHV) from *Penaeus monodon*. Dis. Aquat. Org. 492, 27–34.

Sithigorngul, P., Rukpratanporn, S., Longyant, S., Chaivisuthangkura, P., Sithigorngul, W., Menasveta, P., 2002. Monoclonal antibodies specific to yellow-head virus (YHV) of *Penaeus monodon*. Dis. Aquat. Org. 49, 71–76.

Sittidilokratna, N., Hodgson, R.A.J., Cowley, J.A., Jitrapakdee, S., Boonsaeng, V., Panyim, S., Walker, P.J., 2002. Complete ORF1b-gene sequence indicates yellow head virus is an invertebrate nidovirus. Dis. Aquat. Org. 50, 87–93.

Sittidilokratna, N., Petchampai, N., Boonsaeng, V., Walker, P.J., 2006. Structural and antigenic analysis of the yellow head virus nucleocapsids protein. Virus Res. 116, 21–29.

Sittidilokratna, N., Dangtip, S., Cowley, J.A., Walker, P.J., 2008. RNA transcription analysis and completion of the genome sequence of yellow head nidovirus. Virus Res. 136, 157–165.

Soowannayan, C., Flegel, T.W., Sithigorngul, P., Slater, J., Hyatt, A., Crameri, S., Wise, T., Crane, M.St.J., Cowley, J.A., McCulloch, R., Walker, P.J., 2003. Detection and differentiation of yellow head complex viruses using monoclonal antibodies. Dis. Aquat. Org. 57, 193–200.

Spann, K.M., Cowley, J.A., Walker, P.J., Lester, R.J.G., 1997. Gill-associated virus from cultured *Penaeus monodon* from Queensland, Australia. Dis. Aquat. Org. 31, 169–179.

Walker, P.J., Cowley, J.A., Spann, K.M., Hodgson, R.A.J., Hall, M.R., Withyachumnarnkul, B., 2001. Yellow head complex viruses: transmission cycles and topographical distribution in the Asia-Pacific region. In: Browdy, C.L., Jory, D.E. (Eds.), The New Wave: Proceedings of the Special Session on Sustainable Shrimp Culture, Aquaculture 2001. World Aquaculture Society, Baton Rouge, pp. 227–237.

Walker, P.J., Bonami, J.R., Boonsaeng, V., Chang, P.S., Cowley, J.A., Enjuanes, L., Flegel, T.W., Lightner, D.V., Loh, P.C., Snijder, E.J., Tang, K., 2005. Roniviridae. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), Virus Taxonomy, VIIIth Report of the ICTV. Elsevier/Academic Press, London, pp. 973–977.

Wang, Y.C., Chang, P.S., 2000. Yellow head virus in the giant tiger prawn *Penaeus monodon* cultured in Taiwan. Fish Pathol. 35, 1–10.

Wijegoonawardane, P.K.M., Cowley, J.A., Phan, T., Hodgson, R.A.J., Nielsen, L., Kiatpathomchai, W., Walker, P.J., 2008a. Genetic diversity in the yellow head nidovirus complex. Virology 380, 213–225.

Wijegoonawardane, P.K.M., Cowley, J.A., Walker, P.J., 2008b. Consensus RT-nested PCR detection of yellow head complex genotypes in penaeid shrimp. J. Virol. Methods 153, 168–175.

Wongteerasupaya, C., Vickers, J.E., Sriurairatana, S., Nash, G.L., Akrajamorn, A., Boonsaeng, V., Panyim, S., Tassanakajon, A., Withyachumnarnkul, B., Flegel, T.W., 1995a. A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn *Penaeus monodon*. Dis. Aquat. Org. 21, 66–77.

Wongteerasupaya, C., Sriurairatana, S., Vickers, J.E., Akrajamorn, A., Boonsaeng, V., Panyim, S., Tassanakajon, A., Withyachumnarnkul, B., Flegel, T.W., 1995b. Yellow-head virus of *Penaeus monodon* is an RNA virus. Dis. Aquat. Org. 22, 45–50.

Wongteerasupaya, C., Tongchuea, W., Boonsaeng, V., Panyim, S., Tassanakajon, A., Withyachumnarnkul, B., Flegel, T.W., 1997. Detection of yellow head virus of *Penaeus monodon* by RT-PCR amplification. Dis. Aquat. Org. 31, 181–186.

Wyers, F., Richard-Molard, C., Blondel, D., Dezelee, S., 1980. Vesicular stomatitis virus growth in *Drosophila melanogaster* cells: G protein deficiency. J. Virol. 33, 411–422.