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Consensus RT-nested PCR detection of yellow head complex genotypes in penaeid shrimp

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A consensus RT-nested (n)PCR is described that detects the six distinct genotypic variants in the yellow head virus (YHV) complex. The PCR primers targeted ORF1b gene regions more highly conserved amongst the reference strains of YHV (genotype 1) and gill-associated virus (GAV, genotype 2) and a set of 57 field isolates containing multiple representatives of each genotype. The test employed short PCR (359 bp) and nPCR (147 bp) amplicons to minimise the effects of RNA degradation. To ensure ≤8-primer degeneracy, two primers were designed to each site, one accommodating sequence variations amongst genotype 1 isolates and the other variations amongst isolates of the other genotypes. The analytical sensitivity limits of the PCR and nPCR were estimated to be ~1250 and ~1.25 RNA copies, respectively. The superior group-specificity of the consensus RT-nPCR compared to other OIE-recommended PCR tests for YHV/GAV was demonstrated using RNA from 17 *Penaeus monodon* shrimp infected with representatives of each of the six genotypes. Phylogenetic analysis using the 94 nt ORF1b gene sequence spanned by the nPCR primers generated genotype assignments that were consistent with those obtained using the extended 671 nt sequence used for the initial identification of genotypes.

1. Introduction

Yellow head virus (YHV) from Thailand and gill-associated virus (GAV) from Australia are closely related nidoviruses that are each classified as the type species *Gill-associated virus* of the genus *Okavirus* in the family *Roniviridae* (Cowley et al., 1999; Walker et al., 2005). GAV and YHV primarily infect *P. monodon* shrimp in which they can cause disease and mass mortalities in aquaculture ponds (Boonyaratpalin et al., 1993; Spann et al., 1997). Other similar viruses have been identified in *P. monodon* shrimp from Vietnam (Phan, 2001; Walker et al., 2001) and Thailand (Soowannayan et al., 2003). A recent study of *P. monodon* shrimp samples collected from sites throughout the Indo-Pacific region has identified that YHV and GAV represent two of at least six distinct genetic lineages (genotypes) that comprise the YHV complex (Wijegoonawardane et al., in press). There is evidence that yellow head complex viruses occur commonly as unapparent life-long infections in shrimp (Chantanachookin et al., 1993; Spann et al., 1995; Walker et al., 2001) and are transmitted vertically (Cowley et al., 2002). However, of the six genotypes identified to date, only genotype 1 (YHV) has been detected in shrimp displaying typical signs of yellow head disease (YHD), a devastating OIE-listed disease that results commonly in total crop loss within days of the first appearance of clinical signs in a pond (Limsuwan, 1991; Chantanachookin et al., 1993; OIE, 2006). Genotype 2 (GAV) has been associated with a less severe condition in Australia described as mid-crop mortality syndrome (MCMS), and is far less virulent than YHV (~10^6-fold) in experimental bioassays (Spann et al., 1997; Sittidilokratna and Walker, unpublished data). The other genotypes appear to be widespread in *P. monodon* shrimp throughout the Indo-Pacific region but have not been associated with farm disease outbreaks (Wijegoonawardane et al., in press).

YHV infection and disease can be diagnosed presumptively by gross signs and histopathology (Flegel et al., 1997; OIE, 2006; Tang and Lightner, 1999). However, typical signs of YHD do not always occur and histological observations are not definitive (Sithigorngul et al., 2002; Tang et al., 2002) as similar lesions and histopathology are induced by other viruses (Hasson et al., 1999; Pantoja and Lightner, 2003). A range of molecular methods to detect YHV and/or GAV have been described. Antibody-based detection methods include western blotting (Nadala et al., 2000), dot-blot nitrocellulose enzyme immunoassays (Nadala and Loh, 2000; Sithigorngul et al., 2000, 2002; Intorasoot et al., 2007), immunohistchemistry (Soowannayan et al., 2003), ELISA (Munro and Owens,

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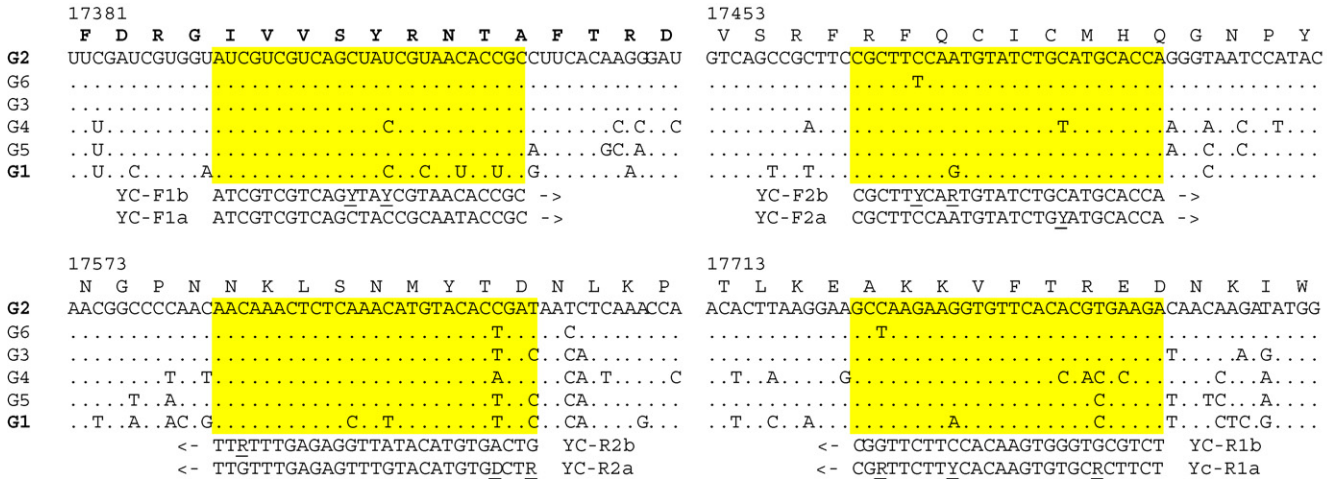

Fig. 1. ClustalX alignment of regions in the 671 nt ORF1b sequence spanned by PCR primer pair YH31-F2:YH31-R2 of reference YHV (G1) and GAV (G2) isolates as well as representative isolates of genotypes 3–6 (G3, G4, G5 and G6). The deduced amino acid sequence of GAV is shown and regions targeted by the consensus RT-nPCR primers YC-F1a/F1b, YC-F2/F2b, YC-R2a/R2b and YC-R1a/R1b are shaded. The positions of the first nucleotide of each block determined from the complete GAV genome sequence (GenBank Accession: AF227196) are shown.

2006) and immunochromatographic test strips (Sithigorngul et al., 2007). Whilst monoclonal antibody-based methods have a capacity to detect YHV specifically and to discriminate other genotypes (Soowannayan et al., 2003), they lack the sensitivity to detect low-level infections and are too cumbersome for routine screening and surveillance applications. *In situ* RNA hybridisation can detect tissues infected with YHV and/or GAV at similar sensitivity to antibody-based methods, but it lacks the specificity to discriminate between genotypes (Tang and Lightner, 1999; Tang et al., 2002; Spann et al., 2003). PCR-based detection offers advantages in speed, sensitivity and specificity, and several single-step (Wongteerasupaya et al., 1997), nested (Cowley et al., 1999) and multiplexed tests (Cowley et al., 2004) as well as quantitative real-time RT-PCR methods using either a TaqMan probe (de la Vega et al., 2004) or SYBR Green chemistry (Dhar et al., 2002; Mouillesseaux et al., 2003) have been developed to detect YHV and/or GAV. However, the recent observation that the yellow head complex comprises at least six distinct genotypes (Wijegoonawardane et al., in press) presents a need to assess current PCR-based methods for specificity of detection and to develop new methodologies for detection and discrimination of all known genotypes.

In this paper, a consensus RT-nested (n)PCR test is described that detects the six currently recognised genotypes in the YHV complex. Primers were targeted to sequences in a 671 nt ORF1b gene region found to be highly conserved in alignments of YHV and GAV reference strains, as well as 57 other isolates that included representatives of the four new genotypes (Wijegoonawardane et al., in press). The new consensus RT-nPCR, combined with amplicon sequence analysis, was found to be far superior to other RT-PCR tests currently listed in the OIE Manual of Diagnostic Tests for Aquatic Animals (OIE, 2006) by providing both group specificity of detection and genotype assignment of viruses in the yellow head complex.

2. Materials and methods

2.1. Primers

Based on a ClustalX multiple alignment of a 671 nt ORF1b gene region 3′-proximal to the helicase domain, PCR primers were designed to sequences more highly conserved amongst the reference Thai YHV (THA-98-Ref, genotype 1) and Australian GAV (AUS-96-Ref, genotype 2) strains and 57 other isolates including representatives of these and YHV complex genotypes 3, 4, 5 and 6 (Wijegoonawardane et al., in press). A sequence alignment of representative isolates of each genotype displaying the regions targeted by the primers is shown in Fig. 1. Both the PCR (359 bp amplicon) and nPCR primers (147 bp amplicon) targeted sequences encoding stretches of amino acids with more limited codon flexibility. In addition, the 3′-termini of forward and reverse primers were terminated at central and first codon positions, respectively, to avoid wobble positions with a greater propensity for change amongst virus isolates. To minimise degeneracy and ensure that a higher proportion of each primer would have good specificity for each genotype compared to that achievable using single more degenerate primers, two primers were designed to each locus. Each of the pair accommodated sequence divergence either amongst genotype 1 isolates ('a' primers) or amongst isolates of the other five genotypes ('b' primers). The primer sequences are shown in Table 1. Individual primers possessed a maximum of 8-fold degeneracy and were designed to have annealing temperatures and sequence compatibilities that conformed closely to the default parameters of the Primer Express 2.0 software (PE Applied Biosystems).

2.2. RNA isolation and YHV complex consensus RT-nPCR

Total RNA was isolated from shrimp lymphoid organ tissue, gill tissue or whole shrimp postlarvae using TRIzol reagent (Ambion), resuspend in 25 μl DEPC-treated water and stored at −80 °C. A small aliquot of the RNA was quantified by UV spectrophotometric determination of absorbance at A_{260nm}.

For cDNA synthesis, 2.0 μg shrimp total RNA, 50 ng random hexamer primers, 1.0 μl 10 mM dNTP mix and sterile DEPC-treated water (14 μl final volume) were incubated at 65 °C for 5 min and chilled on ice. 5 × Superscript III buffer (Invitrogen) (4.0 μl), 1.0 μl 100 mM DTT, 1.0 μl 40 U/μl RNaseOUT (Invitrogen) and 1.0 μl 200 U/μl Superscript III reverse transcriptase (Invitrogen) were then added, followed by gently mixing and incubation at 25 °C for 5 min and 42 °C for 55 min. The reaction was stopped by heating at 70 °C for 15 min, chilled on ice and either used directly or stored at −20 °C.

In the PCR, 1 μl cDNA was added to a reaction mixture (25 μl final volume) containing 1 × *Taq* buffer (10 mM Tris–HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100), 1.5 μl 25 mM $MgCl_2$, 0.35 μl primer mix containing 25 μM each primer YC-F1a, F1b, R1a and R1b, 0.5 μl