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Homologous genetic recombination in the yellow head complex of nidoviruses infecting *Penaeus monodon* shrimp

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ABSTRACT

Yellow head virus (YHV) is a highly virulent pathogen of *Penaeus monodon* shrimp. It is one of six known genotypes in the yellow head complex of nidoviruses which also includes mildly pathogenic gill-associated virus (GAV, genotype 2) and four other genotypes (genotypes 3–6) that have been detected only in healthy shrimp. In this study, comparative phylogenetic analyses conducted on replicase- (ORF1b) and glycoprotein- (ORF3) gene amplicons identified 10 putative natural recombinants amongst 28 viruses representing all six genotypes from across the Indo-Pacific region. The ~4.6 kb genomic region spanning the two amplicons was sequenced for three putative recombinant viruses from Vietnam (genotype 3/5), the Philippines (genotype 5/2) and Indonesia (genotype 3/2). SimPlot analysis using these and representative parental virus sequences confirmed that each was a recombinant genotype and identified a recombination hotspot in a region just upstream of the ORF1b C-terminus. Maximum-likelihood breakpoint analysis predicted identical crossover positions in the Vietnamese and Indonesian recombinants, and a crossover position 12 nt upstream in the Philippine recombinant. Homologous genetic recombination in the same genome region was also demonstrated in recombinants generated experimentally in shrimp co-infected with YHV and GAV. The high frequency with which natural recombinants were identified indicates that genetic exchange amongst genotypes is occurring commonly in Asia and playing a significant role in expanding the genetic diversity in the yellow head complex. This is the first evidence of genetic recombination in viruses infecting crustaceans and has significant implications for the pathogenesis of infection and diagnosis of these newly emerging invertebrate pathogens.

Introduction

The yellow head complex of nidoviruses comprises yellow head virus (YHV), gill-associated virus (GAV) and at least four other closely related genotypes that occur commonly in the giant tiger shrimp (*Penaeus monodon*) in the Indo-Pacific region (Walker et al., 2001; Soowannayan et al., 2003; Wijegoonawardane et al., 2008a). YHV (designated genotype 1) was first reported as the cause of mass mortalities in shrimp ponds in Thailand in 1990 (Limsuwan, 1991) and has since been reported in other shrimp farming regions in Southeast and East Asia (Walker et al., 2001). It is a highly virulent pathogen that can cause total crop loss within several days of the first appearance of disease (Boonyaratpalin et al., 1993; Chantanachookin et al., 1993). GAV (genotype 2) was first observed as a persistent low-level infection in healthy *P. monodon* shrimp in Australia in 1994 (Spann et al., 1995). Although far less virulent than YHV, GAV causes mortalities following experimental infection of shrimp and has been associated with a slowly progressing disease called mid-crop mortality syndrome (MCMS) (Spann et al., 1997, 2003; Callinan and Jiang, 2003). The prevalence of GAV infection in healthy *P. monodon* in eastern Australia approaches 100% and it occurs commonly in healthy shrimp from Vietnam and Thailand (Walker et al., 2001; Wijegoonawardane et al., 2008a). The four other known genotypes in the yellow head complex also occur commonly in healthy *P. monodon* with a distribution that extends from Mozambique in the west through South, Southeast and East Asia (Wijegoonawardane et al., 2008a). None of these four genotypes has yet been associated with disease.

The yellow head complex viruses are enveloped, rod-shaped, (+) sense RNA viruses that are classified as a single species (*Gill-associated virus*) in the genus *Okavirus*, family *Roniviridae*, order *Nidovirales* (Walker et al., 2004). The 26,662 nt YHV genome contains four long open reading frames (ORFs). ORF1a encodes a large polyprotein

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(pp1a) containing *cis*-acting papain-like protease (PLP) and 3C-like protease (3CLP) domains (Sittidilokratna et al., 2008). ORF1b overlaps ORF1a and is expressed only as a result of a (−1) ribosomal frameshift at a 'slippery' sequence upstream of a predicted pseudoknot structure in the overlap region. The extended polyprotein (pp1ab) contains 'SDD' RNA-dependent RNA polymerase, helicase, metal-ion-binding, exonuclease, uridylate-specific endoribonuclease and ribose-2′-*O*-methyl transferase domains of the replication complex (Sittidilokratna et al. 2002, 2008). ORF2 encodes the nucleoprotein (p20) which is the only polypeptide component of the helical nucleocapsid (Soowannayan et al., 2003; Sittidilokratna et al., 2006). ORF3 encodes a polyprotein (pp3) that is processed at signal peptidase type 1 cleavage sites to generate the two virion envelope glycoproteins gp116 and gp64, and an N-terminal triple-membrane-spanning fragment of unknown function (Jitrapakdee et al., 2003). The 26,235 nt GAV genome is smaller, primarily due to significant deletions in intergenic regions (IGRs), but shares ~79% overall nucleotide sequence identity with YHV (Cowley and Walker, 2002; Sittidilokratna et al., 2008). The GAV genome organisation is similar to YHV and all identified functional domains are preserved, but GAV contains an additional small open reading frame near the 3′-terminus (ORF4, 83 aa) that may be expressed at low levels in infected cells (Cowley et al., 2000; Cowley and Walker, 2002; Cowley et al., 2004a; Cowley and Walker, 2008). Like other nidoviruses, YHV and GAV transcribe a nested set of 3′-coterminal, polyadenylated, genomic and sub-genomic mRNAs (Cowley et al., 2002; Sittidilokratna et al., 2008). Partial genome sequence analysis of genotypes 3, 4 and 5 has indicated that they share a similar genome organisation and transcription strategy and are more closely related in sequence to GAV than to YHV (Wijegoonawardane et al., 2008a).

In this paper, we report evidence of natural, high frequency genetic recombination between genotypes in the YHV complex and demonstrate that recombinants can be recovered from mixed experimental infections of shrimp with YHV and GAV. Partial sequence analysis of selected recombinant genomes identified that recombination occurs preferentially at a hotspot near the C-terminus of the ORF1b coding region, suggesting modular functional separation of the structural and non-structural domains. This is the first evidence of genetic recombination in viruses infecting shrimp and has significant implications for the pathogenesis of infection and diagnosis of these newly emerging invertebrate pathogens.

Results

Phylogenetic analyses using ORF1b and ORF3 amplicons

Twenty-eight virus samples (Table 1), representing each of the six identified genotypes in the yellow head complex, were selected from a set of 57 viruses detected previously in shrimp collected from countries across the Indo-Pacific region (Wijegoonawardane et al., 2008a). Total RNA extracted from each of the samples was used as a template for RT-PCR amplification of two genomic regions. The first (668–671 nt), located in ORF1b overlapping and immediately downstream of the helicase domain, was used previously to assign genotypes (Wijegoonawardane et al., 2008a, 2008b). The second region (1287 nt in YHV) was located in the N-terminal domain of ORF3, extending from a locus 28 nt downstream of the ORF3 initiation codon, to a site beyond the internal signal peptidase cleavage site at the N-terminus of gp116. ClustalX multiple alignments of nucleotide sequences obtained from each region, and corresponding genome sequences of the YHV (EU487200) and GAV (NC010306) reference strains, were used to construct neighbour-joining phylogenetic trees (Fig. 1).

Phylogenetic analysis of the ORF1b region (Fig. 1A) identified six distinct lineages corresponding to the six genotypes identified in a similar analysis of the larger set of 57 viruses reported previously (Wijegoonawardane et al., 2008a). Genotype 1 comprised five YHV isolates collected from disease outbreak ponds in Thailand, including the Thai YHV reference strain (THA-98-Ref) isolated from Chachoengsao Province, Thailand, in 1998. Genotype 2 included the GAV reference strain (AUS-96-Ref), a second isolate from a disease

Table 1
Penaeus monodon samples selected for phylogenetic analysis in the ORF1b and ORF3 gene regions.

Sample code	Date of collection	Sample origin	Life stage	Tissues for extraction	Health status	Genotype ORF1b	Genotype ORF3
AUS-97-MCMS1	24.04.1997	Queensland, Australia	Adult	Gill	MCMS[a]	2	2
AUS-00-HL2	2000	Queensland, Australia	Adult	Lymphoid organ	Healthy	2	2
IDN-04-H4[b]	23.01.2004	Indonesia	Sub-adult	Muscle	Healthy	3	2
IDN-04-H7[b]	23.01.2004	Indonesia	Sub-adult	Muscle	Healthy	3	2
IDN-04-H10[b]	23.02.2004	Indonesia	Sub-adult	Muscle	Healthy	3	2
IND-02-H5	15.06.2002	Nellore, India	PL-14	Whole	Healthy	4	4
IND-02-H9	15.06.2002	Nellore, India	PL-15	Whole	Healthy	4	4
MOZ-04-H9	13.01.2004	Mozambique	Brooder	Gill	Healthy	6	6
MYS-03-H2[b]	06.06.2003	Malaysia	Sub-adult	Pleopod	Healthy	3	2
MYS-03-H4[b]	06.06.2003	Malaysia	Sub-adult	Pleopod	Healthy	5	2
PHL-03-H8[b]	02.10.2003	Iloilo, Philippines	PL-12	Whole	Healthy	5	3
THA-01-D10	2001	Nakorn Pathori, Thailand	Juvenile	Gill	Diseased[c]	1	1
THA-01-D8	2001	Nakorn Pathori, Thailand	Juvenile	Gill	Diseased[c]	1	1
THA-03-D29	2003	Chachoengsno, Thailand	Juvenile	Gill	Diseased[c]	1	1
THA-03-D30	2003	Chachoengsno, Thailand	Juvenile	Gill	Diseased[c]	1	1
THA-04-H20	28.03.2004	Supanburi, Thailand	PL-20	Whole	Healthy	2	2
THA-03-HA	2003	Thailand	Adult	Gill	Healthy	2	2
THA-03-HB	2003	Thailand	Adult	Gill	Healthy	2	2
THA-03-HG	2003	Thailand	Adult	Gill	Healthy	2	2
THA-03-SG21	2003	Thailand	Sub-adult	Gill	Slow growth	5	5
TWN-03-H9[b]	05.07.2003	Taiwan	Juvenile	Pleopod	Healthy	3	2
TWN-03-H11[b]	05.07.2003	Taiwan	Juvenile	Pleopod	Healthy	3	2
VNM-02-H258[b]	01.02.2002	Nha Trang, Vietnam	PL-12	Whole	Healthy	3	5
VNM-02-H278[b]	06.03.2002	Hon Chong, Vietnam	PL-12	Whole	Healthy	3	5
VNM-02-H264	03.03.2002	Ca Na, Vietnam	PL-12	Whole	Healthy	3	3
VNM-02-H93	04.02.2002	Hon Khoai, Vietnam	PL-10	Whole	Healthy	3	3
VNM-02-H6	04.02.2002	Vietnam	PL	Whole	Healthy	2	2
VNM-02-H5	01.04.2002	Vietnam	Sub-adult	Muscle	Healthy	3	3

[a] Mid-crop mortality syndrome.
[b] Recombinant genotype.
[c] Yellow head disease.