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Infectious hypodermal and hematopoietic necrosis virus (IHHNV)-related sequences in the genome of the black tiger prawn *Penaeus monodon* from Africa and Australia

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Abstract

We found an infectious hypodermal and hematopoietic necrosis virus (IHHNV)-related sequence within the shrimp genome in populations of *Penaeus monodon* from Africa and Australia. IHHNV is a singlestranded DNA virus that has caused severe mortality and stunted growth in penaeid shrimp. Recently, IHHNV-related sequences were found in samples of *P. monodon* from Madagascar and Tanzania. These sequences vary considerably (14 and 8%, respectively) from that of IHHNV found in association with viral epidemics. Laboratory bioassays were carried out with *P. monodon* and *Litopenaeus vannamei* to determine if either of these IHHNV-related sequences is infectious. We used juvenile and adult *P. monodon* containing the virus-related sequences from four geographic regions to generate inocula and tissues for feeding. Specific pathogen free *P. monodon* and *L. vannamei* were used as indicator shrimp. During the 2–4 week bioassays, none of the indicator shrimp showed signs of infection or disease. Results of both PCR assays and histological examination of the indicator shrimp were negative for IHHNV infection, indicating that the Africa type IHHNV-related sequences are not infectious.

With the shrimp containing the Madagascar type IHHNV-related sequence (designated as type A), we performed genome walking at the 3′ end of the virus-related sequence and found that this virus-related sequence is part of the *P. monodon* genome. A fragment of 1.9 kb flanking sequence was cloned and sequenced. Sequence analysis showed that this flanking sequence contains shrimp microsatellite DNA. Also, its translated amino acid sequence was highly similar to a retrotransposon. This result provides molecular evidence that the type A IHHNV-related sequence is shrimp DNA. This sequence was found in the *P. monodon* collected from Africa and Australia.
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Keywords: Infectious hypodermal and hematopoietic necrosis virus (IHHNV)-related sequences; *Penaeus monodon*; Microsatellite DNA; Retrotransposon

1. Introduction

In routine diagnostic screening for shrimp viruses, we found *Penaeus monodon* collected from Africa reacted positively in PCR assays for the presence of infectious hypodermal and hematopoietic necrosis virus (IHHNV), but tissues from these shrimp were not infectious to *Litopenaeus vannamei* in a laboratory bioassay (Tang et al., 2003). This was unusual as IHHNV is known to infect several species of penaeid shrimp. This virus has caused severe mortalities in *L. stylirostris* (Lightner et al., 1983a, 1983b). It is less virulent in *L. vannamei* and *P. monodon* and does not typically cause mortality; but it can result in runt deformity syndrome, a condition characterized by stunting and cuticular deformities (Bell and Lightner, 1984; Kalagayan et al., 1991; Primavera and Quinitio, 2000).

IHHNV is an icosahedral, non-enveloped, parvovirus with a single-stranded, 4.1 kb, DNA genome comprised of three large open reading frames (ORFs) (Bonami et al., 1990; Shike et al., 2000). It is closely related to the mosquito viruses: *Aedes aegypti densovirus* and *Aedes albopictus densovirus*, thus is named Penaeus stylirostris densovirus and placed as a tentative species of the genus *Brevidensovirus* (Fauquet et al., 2005).

The IHHNV has been found in *P. monodon* from SE Asia (Thailand, Taiwan and the Philippines). Two virus-related sequences were found in Africa (Tang et al., 2003). Type A was found in samples from Madagascar and Australia (Krabsetsve et al., 2004), and type B was found in samples from Tanzania. Both types A and B virus-related sequences contain three ORFs

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and have identical replication initiator motifs and NTP-binding and helicase domains to those of the IHHN virus. The similarity indicates that there is a close relationship between the IHHN virus and virus-related sequences found in *P. monodon* from Tanzania, Madagascar, and Australia.

In this report, we demonstrate that both type A and B IHHNV-related sequences are not derived from an infectious virus. Subsequent determination of the flanking sequence of type A indicated that it is part of the host DNA.

2. Materials and methods

2.1. *P. monodon with Africa type IHHNV-related sequences*

Tissues of *P. monodon* that contain IHHNV-related sequences were used as challenge tissues for laboratory bioassays (Table 1). Three adult *P. monodon* (mean weight: 45 g) containing type A sequence were collected in Madagascar in 2001. Two adults (weight: 53 and 90 g) were collected from Tanzania and Mozambique, respectively, in 2004. These two were found to contain both type A and B IHHNV-related sequences (see below). Eleven juveniles (mean weight: 4 g) that contained type A were produced by broodstock originating from Australia during 2004.

2.2. *Determination of IHHNV-related sequence type by PCR and DNA sequencing*

DNA was extracted from either gills or pleopods of shrimp with a High-pure DNA template preparation kit (Roche Bioscience) and amplified with primers IHHNVF/R1 (Table 2) by PCR to generate a 1.2 kb fragment (Tang and Lightner, 2002). The amplicons were purified with a QIAquick PCR purification kit (Qiagen) and directly sequenced using an automatic DNA sequencer, ABI Prism 377 (Applied Biosystems). The type of sequence was determined from alignment with IHHN virus and virus-related sequences (IHHN virus: GenBank AF218266, AY102034; type A virus-related sequence: GenBank AY125423; type B: GenBank AY124937). For samples that exhibited noisy signals in DNA sequencing, the PCR products were ligated with pGEM-T-Easy vector (Promega) and cloned into *Escherichia coli* JM109. We selected 10 clones containing DNA inserts. The amplicons from each clone were purified and sequenced with primer IHHNVR1. The sequences were analyzed as described above.

Table 1
Laboratory bioassays of *Penaeus monodon* tissues containing IHHNV-related sequences using IHHNV-free *P. monodon* and *Litopenaeus vannamei* as indicator shrimp

Origin of *P. monodon* (type of IHHNV-related sequence)	Challenge route (duration of bioassay)	Indicator shrimp (no. of shrimp, mean weight)
Madagascar (type A)	Injection (4 weaks)	*L. vannamei* (10, 2 g)
		P. monodon (15, 1 g)
	Per os (4 weaks)	*L. vannamei* (15, 0.5 g)
		P. monodon (15, 0.5 g)
Tanzania (type A and B)	Injection (4 weaks)	*P. monodon* (20, 1.5 g)
		L. vannamei (20, 1.5 g)
	Per os (3 weaks)	*L. vannamei* (10, 2.0 g)
Australia (type A)	Injection (2 weaks)	*P. monodon* (12, 1.5 g)
		L. vannamei (12, 1.5 g)
	Per os (3 weaks)	*L. vannamei* (10, 2.0g)
Mozambique (type A and B)	*Per os* (3 weaks)	*L. vannamei* (10, 2.0 g)

Table 2
Primers used in genome walking and PCR assays

Primer designation	Sequence of primer
IHHNVF[a]	5′-ATGTGCGCCGATTCAACAAG-3′
IHHNVR1[a]	5′-CTAAGTGACGGCGGACAATA-3′
IHHNV389F[b]	5′-CGGAACACAACCCGACTTTAT-3′
IHHNV389R[b]	5′-GGCCAAGACCAAAATACGAA-3′
MG-GSP1[c]	5′-ACTGGACCGATCTTTATTCCAAAGTGG-3′
MG-GSP2[c]	5′-CAAAGGACCATCCGGAGAAGAACTAAA-3′
MG831F[d]	5′-TTGGGGATGCAGCAATATCT-3′
MG831R[d]	5′-GTCCATCCACTGATCGGACT-3′
PM2R[d]	5′-CTGGTGCCTCAGTAGGTGGT-3′
IHHNV395F[d]	5′-CATTTACAGACACCCCATATTTAGA-3′
IHHNV3605R[d]	5′-TGCCTGGGTAGCTGGTATGTATA-3′
MG280F[d]	5′-GCAGCAATATCTCCATAAACC-3′

[a] Used to determine the type of IHHNV-related sequence.
[b] Used for detection of IHHN virus and IHHNV-related sequences.
[c] Used for genome walking for 3′ end flanking sequence.
[d] Used for detection of type A IHHNV-related sequence in the shrimp genomic DNA.

2.3. *Laboratory bioassays*

We carried out seven laboratory infections, details of these are shown in Table 1. For indicator shrimp, small juveniles of specific pathogen free (SPF) *L. vannamei* (Kona stock) were provided by Oceanic Institute in Hawaii. *P. monodon* were provided by an anonymous shrimp grower in Hawaii; in this population, IHHNV or IHHNV-related sequences were not detected by PCR, and no other signs of infection by the known shrimp viruses were found by histological examination.

The indicator shrimp were exposed to IHHNV-related sequences through injection of tissue homogenate prepared as described in Tang et al. (2003) or by feeding the shrimp minced tissues. For injection bioassays, each shrimp was injected with 0.1 ml of inoculum. For *per os* challenges, the indicator shrimp were fed at 10% of their body weight once daily for 3 days. The shrimp were then maintained for 2–4 weaks on a commercial pelletized ration (Rangen). At the end of bioassays, gill and surrounding soft tissues of challenged shrimp were sampled for IHHNV PCR assay using primers IHHNV389F/R (Table 2). Their cephalothoracies were preserved in Davidson's AFA fixative for histological examination (Bell and Lightner, 1988).

2.4. *Genome walking and sequence analyses*

Total DNA of the adult *P. monodon* from Tanzania was extracted with a High-pure PCR template preparation kit (Roche

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