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Detection of infectious hypodermal and haematopoietic necrosis virus (IHHNV) in farmed Australian *Penaeus monodon* by PCR analysis and DNA sequencing

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ABSTRACT

Infectious hypodermal and haematopoietic necrosis virus (IHHNV) of shrimp has recently been classified as *Penaeus stylirostris brevidensovirus* (PstDNV) in the family Parvoviridae. Genetic variants have been reported in the Black Tiger shrimp *Penaeus monodon* over its natural range from East Africa to Australasia. Previous reports from Australia have suggested that a distinct, non-infectious type of IHHNV that is inserted into the shrimp host genome is the only type of IHHNV that occurs there. Here we describe an infectious type of IHHNV identified from farmed *P. monodon* in Australia in April 2008 by PCR amplification from two regions of the IHHNV genome, one reported to be discriminatory for the infectious type of IHHNV and the other (from IHHNV ORF1/2) not described in the non-infectious, inserted type. In addition, we used overlapping PCR primers to amplify all of the ORFs of the IHHNV genome (approximately 3.6 kb) from the Australian samples, except for the hairpin loop ends (GQ475529). Comparison of the maximum possible portion of this sequence with 8 GenBank records of IHHNV isolates reported from Asia (2814 bases relative to positions 588 to 3413 of GenBank AF273215) revealed 94–95% identity in nucleic acid sequence and 96 to 97% identity in amino acid sequence. These results were in agreement with an official report by the World Organization for Animal Health in July 2008 for the presence of infectious IHHNV in Australia.

1. Introduction

Infectious hypodermal and haematopoietic necrosis virus (IHHNV) is a shrimp virus that has recently been classified as *Penaeus stylirostris brevidensovirus* (PstDNV) in the family Parvoviridae (Tattersall et al., 2005). However, it will be referred to here as IHHNV since that is the name by which it is most commonly known. IHHNV was first described from high mortality disease outbreaks in cultured *Penaeus (Litopenaeus) stylirostris* in the Americas (Lightner et al., 1983). It also infects *Penaeus (Litopenaeus) vannamei*, causing stunted growth and deformities called runt deformity syndrome (RDS) (Bell and Lightner, 1984). It is now believed that IHHNV reported in the first American outbreaks originated from *Penaeus (Penaeus) monodon* imported as aquaculture stocks from Southeast Asia (Lightner, 1996; Tang et al., 2003).

In addition to infectious IHHNV, non-infectious inserts of the partial IHHNV genome into the shrimp genome have recently been discovered in captured *P. monodon* from East Africa and Australia (Krabsetsve et al., 2004; Tang and Lightner, 2006). Since these sequences can give positive test results using the formerly recommended PCR method (Anonymous, 2006), a modified method was developed specifically to detect the infectious type (Tang et al., 2007). Although non-infectious IHHNV has been reported previously from Australia (Krabsetsve et al., 2004; Tang and Lightner, 2006), the infectious type has not, and this has opened the question as to whether it occurs in Australia or not.

In this study we describe positive test results for infectious IHHNV obtained with specimens of farmed *P. monodon* from Australia. Analysis of PCR fragments generated from DNA extracts of these samples indicated that sequences of the infectious Australian isolate had high identity to sequences of IHHNV from nearby areas in Southeast Asia.

2. Materials and methods

2.1. Australian shrimp samples

Fresh raw shrimp samples were collected from a black tiger shrimp (*P. monodon*) farm in northern Queensland, Australia on March 2008. They were frozen and shipped to Thailand where they were stored.

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Forty individual shrimps were removed and thawed for DNA extraction. The 40 shrimps were arbitrarily divided into 8 groups of 5 shrimps each and the gills or pleopods from each group were pooled for DNA extraction. Each pooled extract was used for one PCR reaction.

2.2. DNA isolation from P. monodon from Australia and Thailand

A frozen sample (−80 °C) of DNA extracted from Thai *P. monodon* infected with IHHNV was used as a local reference sample. Total DNA was extracted from gills and pleopods of the Australian shrimp samples using the phenol-chloroform method (Sambrook and Russell, 2001). DNA was quantified by UV spectrophotometry (A_{260} nm).

2.3. PCR detection of IHHNV isolates

Fragments of IHHNV genomic sequences from Australian samples were amplified by PCR using the IHHNV-specific primers listed in Table 1. PCR was conducted in a 30 µL reaction volume containing 3 µL PCR buffer (10×), 1 µL $MgCl_2$ (50 mM), 0.6 µL dNTP (10 mM), 1 µL of each forward and reverse primer (10 mM), 22.2 µL water, 0.2 µL (1U) Taq DNA polymerase, and 1 µL DNA template. The pair of primers, IHHNV279/IHHNV940, was designed from a complete IHHNV genomic sequence (GenBank AF218266) and targeted the nucleotide positions 279–940 for which matching sequences have not been reported from inserted IHHNV Types A and B. The PCR amplification protocol consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 40 s, 55°C for 40 s, and 72°C for 2 min, with a final extension at 72°C for 7 min.

The primers IHHNV309F/R previously reported to discriminate infectious IHHNV from non-infectious types A and B in *P. monodon* (Tang et al., 2007) were also used. The PCR amplification protocol consisted of initial denaturation at 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 50 s, with a final extension at 72°C for 2 min, and the expected amplicon size was 309 bp.

2.4. Nucleotide sequence analysis

Overlapping primers (Table 2) were used by PCR to amplify the complete IHHNV genome (approximately 3.6 kb) from the Australian samples. PCR products were purified using QIAquick columns (QIAGEN), according to the manufacturer's protocol. The amplified DNA fragments were ligated into pJET1.2/blunt cloning vector (Fermentas) and transformed in JM109 competent cells as described in Sambrook and Russell (2001). Sequencing of both strands of each PCR product was carried out by Macrogen, Korea using a BIG DYE 3.1 terminator mix on an ABI 377 Sequencer. ClustalW program (Larkin et al., 2007) was used to align the sequences of our Australian isolate with matching records of isolates of infectious INNHV (GenBank AF218266, AF273215, AY102034, AY355306, AY355307, AY355308, AY362547, AY362548, EF633688) and non-infectious Type A [GenBank EU675312 and DQ228358 (formerly AY125423)] and Type B (GenBank AY124937). Matched DNA sequences of 2814 bases corresponding to positions 559 to 3413 of the GenBank sequence AF273215 were used by an unweighted pair group method with mathematical average (UPGMA) (Ewens and Grant, 2005) to construct a phylogram (cluster tree) with Mega 4 (version 4.0.2) software (http://www.megasoftware.net) (Tamura et al., 2007) set to default parameters. The matching region from the full sequence of *Aedes aegyptii* densovirus (AeDNV) (GenBank NC_012636), also from the genus *Brevidensovirus*, was used as an out-group. From the same matched DNA sequences, a multiple alignment of 666 deduced amino acids corresponding to positions 599 to 2596 of the non-structural gene 1 (NS1) of AF273215 was carried out using the ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

Table 1
Primer sequences used for PCR detection.

Designation	Sequence	Nucleotide start position	Expected amplicon size
IHHNV279	5'-GTGAACCAACAGAAGTCTTTC-3'	279	662
IHHNV940	5'-GGACCTGGGGTGAGAAGGCT-3'	940	
IHHNV309F*	5'-TCCAACACTTAGTCAAAACCAA-3'	1936	309
IHHNV309R*	5'-TGTCTGCTACGATGATTATCCA-3'	2244	

*From Tang et al. (2007).

Table 2
Primers used for amplifying the complete IHHNV genome.

Designation	Sequence	Nucleotide start position	Tm (°C)
ihhnA_F158	5′-ATGGAAGATACGAACAACCAC-3′	158	58.7
ihhnA_R723	5′-GGACCTGGGGTGAGAAGGCT-3′	723	66.5
ihhnA_F702	5′-CAAGCCTTCTCACCCCAGG-3′	702	64.5
ihhnA_R1260	5′-TCACTCTCTTCCAGTCGCCT-3′	1260	62.4
ihhnA_F1059	5′-GAACCAGAAACTCCAACACCA-3′	1059	60.6
ihhnA_R1507	5′-GATATTTCTAACAAGTACCGTAGTCGC-3′	1507	63.1
ihhnA_F1451	5′-GTTACCTTTGCTGCCAGAGC-3′	1451	62.4
ihhnA_R2027	5′-TGTCTGCTACGATGATTATCCA-3′	2027	58.9
ihhnA_F2002	5′-AGCTTGGATAATCATCGTAGCAG-3′	2002	61
ihhnA_R2355	5′-GGAGGTACCCAGTAGTCTATATC-3′	2355	62.8
ihhnA_F2136	5′-GAACAGGAGACTCAAACACCTTCC-3′	2136	64.6
ihhnA_R2569	5′-GGGCTTGCTCTTGTTGAATCGG-3′	2569	64.5
ihhnA_F2544	5′-TGCGCCGATTCAACAAGAGC-3′	2544	62.4
ihhnA_R3100	5′-GCTGTTGATTGTACGGTCACAAG-3′	3100	62.8
ihhnA_F3168	5′-GCCTATACAATCCATGGTGAC-3′	3168	60.6
ihhnA_R3504	5′-CATCTGTTAAGTTGTCCAGT-3′	3504	56.3
ihhnA_R3625	5′-GTATGTATATAGGGTAGGTATAG-3′	3625	57.4
ihhnA_R3759	5′-GACTCTAAATGACTGACTGACG-3′	3759	60.8

3. Results and discussion

The reference DNA from Thai *P. monodon* and 7 out of the 8 groups of pooled frozen *P. monodon* from Australia gave positive PCR results (an amplicon of 309 bp) with primers IHHNV309F/R (Tang et al., 2007) (Fig. 1). According to Tang et al. (2007), a single 309 bp amplicon with this assay indicates the presence of infectious type of IHHNV rather non-infectious types A (DQ228358) or B (AY124937). We compared our Australian sequence (minus the primer sequences = 265 bp) with matching regions of IHHNV sequence records at GenBank and found that it had 98% identity to sequences of infectious IHHNV from Thailand (AY102034) and Taiwan (AY355307) and 97–98% identity to matching sequences from India (EU552487, EU848309 and EU848312). It shared lower identity (92–96%) with several other sequences originating from the Americas (AY362548, AF218266, AF273215), China (EF633688),

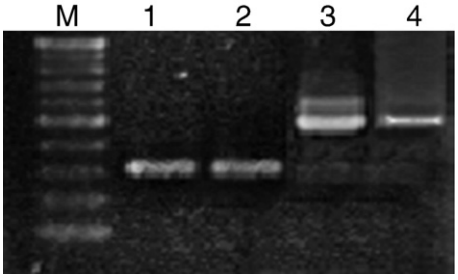

Fig. 1. Example agarose gel showing bands of PCR amplicons obtained using Thai and Australian shrimp samples. Lane M: 2-log DNA ladder. Lane 1 (Thai) and Lane 2 (Australian) samples tested using primers IHHNV309F/R specific for infectious IHHNV. Lane 3 (Thai) and 4 (Australian) samples tested using primers IHHNV209/940.