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Short communication

Prevalence of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in *Penaeus vannamei* cultured in northeastern Brazil

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ABSTRACT

Infectious hypodermal and hematopoietic necrosis virus (IHHNV) is a shrimp disease agent that causes mortalities of up to 90% in *Penaeus stylirostris* but does not cause lethal infections in *Penaeus vannamei*. The purpose of this study was to determine the prevalence of IHHNV in *P. vannamei* cultured in northeastern Brazil, the highest shrimp producing area in the country, by one-step polymerase chain reaction (PCR). Using one-step PCR, we amplified a 185 bp fragment of IHHNV genome and determined IHHNV prevalence on 26 rearing ponds of seven farms. Shrimp from all the ponds showed infection by IHHNV and the prevalence of the virus in the ponds ranged from 9.4% to 81%. Significant differences in IHHNV prevalence among rearing ponds from the same farm were found. These differences might be attributed to different larval origins, handling techniques and environmental factors. Despite the high IHHNV prevalence in shrimp populations, a relationship between cultivation time and gross signs of RDS were not observed. Our results showed that IHHNV infection was common in rearing ponds, but the clinical findings indicated no apparent impact of IHHNV disease on the shrimp, suggesting that *P. vannamei* reared in northeastern Brazil could have the so-called "non-infectious IHHNV form".

1. Introduction

Viral diseases are a serious problem in cultured penaeid shrimp. Infectious hypodermal and hematopoietic necrosis virus (IHHNV) is a shrimp pathogen responsible for 90% of mortality in juveniles *Penaeus stylirostris* (Lightner et al., 1983b), but this virus does not cause lethal infection in *Penaeus vannamei* (Lightner et al., 1983a; Bell and Lightner, 1984). IHHNV is an icosahedral non-enveloped virus, 22 nm in diameter, with a ssDNA genome 4.1 kb in length (Mari et al., 1993). This virus is considered a member of the Parvoviridae family, due to its morphology and biochemical structure (Bonami et al., 1990). More recently, IHHNV was classified into the sub-family *Densoirinae*, genus *Brevidensovirus*, as *Penaeus stylirostris densovirus* (PstDNV) (Tattersall et al., 2005). Infectious hypodermal and hematopoietic necrosis disease was first detected in 1981, in juvenile *P. stylirostris* stocks imported into Hawaii from Costa Rica and Ecuador (Lightner et al., 1983a,b). The virus may have subsequently spread throughout the Americas as a consequence of the movement of host stocks for aquaculture (Lightner, 1996a). IHHNV infection has been reported in other cultured shrimp species such as *Penaeus monodon*, *Penaeus japonicus* and *Penaeus vannamei* (Bell and Lightner, 1984; Lightner et al., 1997). Chronic infection in *P. vannamei*, in addition to retarding growth, causes cuticular deformities of the rostrum, antenna, thoracic and abdominal areas. These collective clinical signs are known as runt deformity syndrome (RDS) (Kalagayan et al., 1991). Shrimp with RDS have variable growth rates, which reduces their market value by 10% to 50%, depending on the level of infection (Lightner and Redman, 1998).

IHHNV infection can occur by horizontal transmission through the ingestion of dead infected shrimp or by contact with water containing infected animals (Lotz, 1997).Vertical transmission has been suggested by the presence of IHHNV in the ovaries of infected *P. vannamei* females (Motte et al., 2003). Thus, horizontal and vertical transmission may increase IHHNV prevalence in cultured shrimp. In this study, one-step PCR was used as the diagnostic method for detecting IHHNV. PCR, one of the most sensitive methods, was applied to determine IHHNV prevalence in rearing ponds. Gross signs of RDS were also investigated in *P. vannamei* penaeid shrimp collected on farms in northeastern Brazil.

2. Materials and methods

2.1. Shrimp samples

Cultured juvenile white shrimp *P. vannamei* were collected from twenty six rearing ponds, on seven farms, denominated F1 to F7, in

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northeastern Brazil. The number of ponds/farm is shown in Table 1 and sampling ranged from 20 to 60 shrimp/pond. According to the equation $n=[1-(1-C)^{(1/I)}][N-I/2]+1$ (www.leb.fmvz.usp.br), n being sample size, I the number of infected individuals, C the confidence level (95%), and N the population size (>100,000), the size of all the samples was sufficient. A total of 1087 specimens were collected on the seven farms. The weight of each shrimp and the cultivation time were recorded. The IHHNV prevalence/pond was determined by one-step PCR and the presence of RDS signs was investigated.

The shrimp were disinfected with 70% ethanol. Approximately 200 µl of hemolymph was collected from the ventral sinus, using a 3-cc syringe containing 200 µl of 10% sodium citrate, then stored at −20 °C until processing. One pleopod from the second pair was collected and kept in 95% ethanol until PCR processing.

2.2. DNA extraction

DNA extraction from hemolymph and pleopods was performed using lysis buffer (Tris 10 mM, EDTA 1 mM, SDS 1%, pH 8.0) and 10 mg/ml proteinase K. Pleopods were homogenized and both tissues were incubated at 65 °C for 1 h. After this time, 5M NaCl and 10% CTAB were added and incubated at 55 °C for 1 h. The DNA was separated from the protein by centrifugation in a chloroform-isoamyl alcohol mixture. DNA was precipitated in isopropanol and the pellet was washed in 70% ethanol. Dried DNA was dissolved in ultrapure water and stored at −20 °C (modified from Sambrook et al., 1989).

2.3. PCR Amplification of IHHNV

One-step PCR was done using extracted DNA from hemolymph and pleopod homogenate as the template. Negative (ultra-pure water) and positive control (from the IHHNV kit, Diagxotics, USA, kindly donated by the Center for Shrimp Diseases-CEDOC-RN-Brazil) were also included. PCR primers were designed based on the published IHHNV genomic sequence (GenBank, accession number AF 218266). Primers amplify a nucleotide sequence located in a region that encodes a non-structural protein (816–2816 nucleotides) and overlaps with a structural protein region (2758-3747 nucleotides). The exact target of the primers 5′-AACAGCCAGTACGA CATCAACC-3′ (sense) and 5′-CGGCGTGTTCTTCGTCTTCATT-3′ (antisense), relative to AF 218266, was 185 nucleotides from position 2652 to 2836. The PCR reaction mixture contained 2.5 µl of DNA; 0.5 µM of each primer; 0.2 µM dNTPs; 3 mM $MgCl_2$; 1x Taq reaction buffer (20 mM Tris–HCl, 50 mM KCl, pH 8.3); 0.5 U Taq DNA polymerase (Invitrogen, São Paulo, Brazil), in a 25 µl final volume reaction. Reagents were covered with mineral oil to prevent evaporation and PCR was performed in an automatic thermocycler (Mastercycler Eppendorf, Hamburg, Germany). The PCR cycling conditions were 95 °C for 5 min, followed by 40 cycles of 95 °C for 1 min, 62 °C for 1 min and 72 °C for 1 min, with a final elongation step at 72 °C for 7 min. A 5 µl PCR product of each sample was analyzed by 8% polyacrylamide gel electrophoresis, with a 100-bp DNA ladder as a marker (Invitrogen, California, USA) and stained by silver nitrate (Sanguinetti et al., 1994). To gauge the success of the extraction process and determine DNA quality, the *P. vannamei* β-actin gene was amplified in parallel with IHHNV DNA. The primers 5′-AGCAAGCGAG GTATCCTCAC-3′ (sense) and 5′-TATCCCTCGTAGATGGGCAC-3′ (antisense) for the *P. vannamei* β-actin gene were selected from a published sequence (accession no. AF300705, GenBank). To confirm that the PCR fragment observed by electrophoresis was specific to IHHNV, the PCR product was sequenced using DYEnamic ET Dye Terminator Cycle Sequencing kit for MegaBACE DNA Analysis Systems (Amersham Bioscience). A search for significant similarities between the amplified fragment and IHHNV sequences in GenBank was performed using BLAST at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

Table 1
Prevalence of IHHNV, cultivation time, weight and the coefficient of weight variation in *P. vannamei* cultured in the ponds of seven farms in northeastern Brazil

Farm/ponds number	Pond	Shrimp (*n*)	Time of cultivation (days)	Mean weight± SD (g)	Coefficient of weight variation (%)	IHHNV prevalence (%)
F5/7	1	29	73	6.3±1.37	21.7	69.0*
	2	27	98	8.5±1.47	17.3	55.6*
	3	59	102	8.9±0.57	6.4	52.5*
	4	30	48	7.5	NA	20.0
	5	30	57	9.2	NA	23.3
	6	30	90	12	NA	36.7*
	7	58	56	8.9±0.54	6.1	72.4*
F7/9	8	46	97	9.6±0.48	5.0	26.1
	9	57	132	13.4±0.6	4.5	73.7*
	10	49	163	16.7	NA	30.6*
	11	56	113	9.8±1.41	14.4	17.9
F1/20	12	32	72	10.8	NA	46.9*
	13	20	74	10.3	NA	70.0*
	14	20	91	14.4±0.41	2.8	50.0*
	15	45	54	12.6±0.34	2.7	31.1
	16	50	88	14.2±0.34	2.4	22.0
F4/12	17	48	69	7.9±0.71	9.0	62.5*
	18	60	83	9.4±1.12	11.9	50.0
	19	22	97	12	NA	54.6
F6/7	20	28	49	4.0	NA	17.9
	21	48	90	9.4±0.81	8.6	47.9*
	22	58	124	12.6±1.7	13.4	81.0*
	23	53	108	11.8±2.3	19.5	9.4
F2/2	24	60	90	5.6±0.63	11.3	30.0*
	25	29	153	8.5	NA	17.2
F3/34	26	43	125	6.2±1.19	19.2	18.6

*Indicates ponds with IHHNV prevalence significantly higher than the lower prevalence of the same farm ($P<0.05$).
NA (not applicable) for coefficient of weight variation in ponds with only the mean weight of shrimp.

2.4. Gross signs of RDS

Shrimp were collected in rearing ponds and gross signs characteristic of RDS (e.g. high variation in body size, dark body color, soft shell and distorted rostra and antennal flagellae), were searched (Kalagayan et al., 1991; Primavera and Quinitio, 2000).

2.5. Statistical analysis

The different IHHNV prevalences among rearing ponds on the same farm were analyzed by binomial test (BioEstat 4.0, 2004), a non-parametric test based on binomial distribution of dichotomous dependent variables (infection versus non-infection), compared to the probability of the occurrence of higher prevalence than the lowest prevalence found on the farm.

The Multiple Logistic Regression test (Statistica 7.1 software) was used to analyze the effect of cultivation time on the IHHNV prevalence and the effect of this prevalence on shrimp weight.

3. Results

3.1. IHHNV diagnosis by one-step PCR

Under standardized one-step PCR conditions using specific primers, a single 185 bp product was amplified from IHHNV genome (Fig. 1). Nucleotide sequencing confirmed that the 185 bp fragment obtained by one-step PCR was specific to the IHHNV genome. Sequencing showed 56 nucleotides with good legibility from the 185 bp PCR fragment. This 56-nucleotide sequence had a 98% identity with respect to the five IHHNV sequences available in the BLAST program, GenBank (EF 633688.1, AY 355308.1, AY 355306.1, AY 362548.1, AF 218266.1), confirming that the 185 bp fragment obtained by one-step PCR was specific to the IHHNV genome. A PCR for the β-