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Use of Polymerase Chain Reaction for the Detection of Infectious Hypodermal and Hematopoietic Necrosis Virus in Penaeid Shrimp

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Abstract: A rapid and reliable polymerase chain reaction (PCR) method was developed for the detection of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in penaeid shrimp. The oligonucleotide primers amplify a 1681-bp fragment of IHHNV, which encompasses the coding sequence for one of the viral coat proteins. The PCR method detects IHHNV in hemolymph and homogenized tissue obtained from the cephalothorax or pleopods of infected shrimp. The technique was also successfully applied to tissue samples preserved in 70% ethanol. The correct size fragment was amplified using IHHNV obtained from six different geographic regions in three different species of penaeid shrimp. No DNA extraction method was necessary for this technique. The use of hemolymph or pleopods provides a nondestructive screening method by which to test juveniles and adult broodstock for the presence of IHHNV.

Key words: PCR, IHHNV, penaeid, shrimp.

INTRODUCTION

Infectious hypodermal and hematopoietic necrosis (IHHN) is one of the major viral diseases of cultured penaeid shrimp. The disease was first detected in 1981 in *Penaeus stylirostris* imported into Hawaii from commercial hatcheries in Costa Rica and Ecuador (Lightner et al., 1983a, 1983b). The disease was highly lethal in *P. stylirostris* juveniles and resulted in mortalities of up to 90% in affected populations. The virus was subsequently found to be widely distributed in culture facilities in North, South, and Central America, the Caribbean, and Central and IndoPacific (Lightner et al., 1992a). In late 1987, IHHNV was documented in *P. stylirostris* imported into Mexico where it spread throughout culture facilities along the Sea of Cortez (Gulf of California) within a period of 3 years (Lightner et al., 1992b). Of notable importance is the spread of the virus into the wild populations of penaeid shrimp in the Sea of Cortez since 1987 (Pantoja et al., 1999; Morales-Covarrubias et al., 1999).

Early studies of this virus demonstrated, by bioassay with susceptible *P. stylirostris,* that *Penaeus vannamei* could be infected by IHHNV, but the virus did not cause the serious mortalities that were typically seen in *P. stylirostris* (Lightner et al., 1983a). Subsequently, Kalagayan et al. (1991) linked IHHNV infection to runt deformity syndrome (RDS) in *P. vannamei.* As the name implies, RDS causes stunting and a large variation in size when *P. van-*

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namei are reared for maximum growth in production facilities. The disease reduces count size (number per pound or kilogram) and, therefore, the market value of infected populations. Nonetheless, *P. vannamei* is still considered to be relatively resistant to the lethal effects of the virus. Naturally acquired infections with IHHNV have been observed in several other commercially important penaeid shrimp species, *Penaeus monodon, Penaeus semisulcatus,* and *Penaeus japonicus* (Lightner et al., 1992b). For several reasons that include the capacity of the virus to cause serious epizootics in *P. stylirostris* and infect other penaeid species, as well as the history of its accidental spread to wild populations of shrimp in the Sea of Cortez, IHHNV has been listed as an economically significant pathogen by the Gulf Coast Research Marine Shrimp Farming Consortium (Lotz et al., 1995). This listing as a reportable pathogen is also carried by the Office International Des Épizooties (OIE; Lightner et al., 1997). The purpose of these listings is the development and maintenance of specific pathogen free (SPF) populations of penaeid shrimp (Lotz et al., 1995).

The diagnosis of infection by IHHNV has been based on the demonstration of key histological features of this disease, especially the prominent eosinophilic intranuclear inclusion bodies surrounded by marginated chromatin within hypertrophied nuclei of infected cells. These characteristic Cowdry type A inclusion bodies are evident in tissues of both ectodermal origin (gills, epidermis, nerve cord, and nerve ganglia) and mesodermal origin (hematopoietic organ, antennal gland, gonads, lymphoid organ, connective tissue, and striated muscle) using hematoxylin and eosin (H&E) staining of tissues fixed in Davidson's AFA (alcohol, formaldehyde, and acetic acid) (Bell and Lightner, 1988, Lightner, 1996). Bonami et al. (1990) successfully purified and characterized the etiological agent of IHHN as an icosahedral unenveloped virus, 22 nm in diameter, with a genome consisting of a single-stranded linear DNA molecule approximately 4.1 kb in size. This virus closely resembles members of the family Parvoviridae, subfamily Densovirinae. Subsequently, in 1993, the genome of IHHNV was cloned and genomic probes were developed that greatly improved the detection methods for the virus (Mari et al., 1993). In situ hybridization, with highly specific and sensitive digoxigenin (DIG)–labeled probes for IHHNV, stain the Cowdry type A inclusion bodies with a purple precipitate. The use of DIG-labeled DNA probes in dot blot and in situ hybridization assays, with fresh tissues and histological specimens, has greatly simplified the diagnosis of the disease (Lightner, 1996).

Using DNA sequence data derived from the cloned library (Mari et al., 1993), several oligonucleotide primers were identified for development of a rapid detection method using the polymerase chain reaction (PCR) technology (Mullis and Faloona, 1987). This report describes the development and testing of a PCR method for the detection of IHHNV in penaeid shrimp.

Materials and Methods

Samples Tested

For this study, three types of *Penaeus* sp. samples were tested for the presence of the IHHN virus. These included frozen tissue samples stored at −70°C, hemolymph samples frozen at −20°C, and pleopods preserved in 70% ethanol at room temperature. The penaeid species examined included *P. stylirostris, P. vannamei,* and *P. monodon.* Six different geographic isolates of IHHNV were used for these studies (Table 1), originating from the United States (Hawaii and Texas), Mexico, Panama, Ecuador, and the Philippines. Most samples tested had naturally acquired IHHNV infections, which were confirmed by routine H&E staining of Davidson's fixed samples or by in situ hybridization with a specific DIG-labeled IHHNV gene probe (DiagXotics, Wilton, CT, Conn.). To experimentally induce IHHNV infection, shrimp were fed minced IHHNV-infected tissues at a rate of 10% of the body weight of the shrimp once a day for 3 consecutive days. They were then fed standard pelleted feed for a period of 4 to 6 weeks, at which time hemolymph was collected and the tissue was either frozen or fixed in 70% ethanol or in Davidson's AFA. A preparation of purified IHHNV served as a positive control in PCR assays. Purification of IHHNV from tissue was performed as described by Bonami et al. (1990). The shrimp used for purification were naturally infected *P. stylirostris* from Mexico. In addition, hemolymph was obtained from experimentally induced infections using two other penaeid shrimp viruses, Taura syndrome virus (TSV) and white spot syndrome virus (WSSV; Lightner, 1996). These samples were used to test specificity of the PCR primers. A population of SPF *P. vannamei* obtained from the Oceanic Institute in Hawaii was used as the source of uninfected hemolymph and tissue and served as a negative control for PCR reactions. Plasmid DNA from clone BA401 was purified using Qiagen-500 columns (Qiagen, Inc., Chatsworth, Calif.) according to the manufacturer's directions. The concentration of the plas-

Table 1. Shrimp Species and Viral Isolates Used for Polymerase Chain Reaction Detection of IHHNV

Year collected	Shrimp species	Virus present	Geographic origin of virus*	Type of infection†
1987	*P. stylirostris*	IHHNV	Hawaii	Natural
1992	*P. stylirostris*	IHHNV	Ecuador	Natural
1993	*P. vannamei*	IHHNV	Texas	Natural
1995	*P. stylirostris*	IHHNV	Mexico	Natural
1996	*P. stylirostris*	IHHNV	Mexico	Natural
1997	*P. vannamei*	IHHNV	Panama	Natural
1996	*P. monodon*	IHHNV	Philippines	Natural
1996	*P. stylirostris*	IHHNV	Philippines	Experimental
1997	*P. stylirostris*	IHHNV	Hawaii	Experimental
1998	*P. stylirostris*	IHHNV	Hawaii	Experimental
1995	*P. vannamei*	WSSV	China	Experimental
1998	*P. vannamei*	TSV	Hawaii	Experimental
1998	*P. vannamei*	None-SPF	NA	NA

*Origin of the IHHNV-infected shrimp (natural) or origin of the infected tissue used to induce infection in the laboratory (experimental).
†Shrimp used had naturally occurring IHHNV or were experimentally infected with IHHNV. NA indicates not applicable.

mid DNA was obtained from optical density readings taken at a wavelength of 260 nm (Maniatis et al., 1982).

Sample Processing

Frozen *Penaeus* sp. tissues, pleopods or the gnathothorax with the carapace and hepatopancreas removed, were homogenized using a Potter (glass/Teflon) tissue blender, in a solution of TN buffer (0.02 M Tris-HCl, 0.4 M NaCl, pH 7.4) at the rate of 1 g of tissue to 10 ml of buffer. Homogenized samples were centrifuged for 10 minutes at 650 *g*. The resulting supernatant fluid was used in the PCR reaction. Pleopods that had been fixed in 70% ethanol were first rinsed in TN buffer. The soft tissue was excised from the outer carapace and homogenized using the procedure described for frozen tissue. Sodium citrate (10 µl of 10% solution per 300-µl sample) was used to prevent coagulation of hemolymph, which was drawn from the ventral sinus. The hemolymph was then used directly in the PCR reaction. In some cases, the hemolymph was boiled for 10 minutes and centrifuged briefly to pellet the coagulated protein, and the clear supernatant fluid was used in the PCR reaction. Samples fixed in Davidson's AFA were processed for routine H&E histology to confirm infections in experimental animals (Bell and Lightner, 1988; Lightner, 1996).

DNA Primers

Two oligonucleotides (designated 2553 and 77012) were synthesized at the University of Arizona, Arizona Research Laboratory, Division of Biotechnology Macromolecular Structures Facility, using a 4-column ABI 394A DNA synthesizer. Each primer, containing 18 nucleotides, was chosen to amplify a 1681-bp segment of the IHHNV genome (GenBank accession number AF218266). This IHHNV fragment will hereafter be referred to as the 1.6-kb amplified product. The primer sequences of 2553 and 77012 are 5′-CGGACAATATCCCTGACT-3′ and 5′-ATCGGTGCACTACTCGGA-3′, respectively. The lyophilized oligonucleotide primers were resuspended in sterile water at a stock concentration of 1 µg/µl and stored at −70°C.

PCR Amplification

The PCR experiments using shrimp tissue homogenate or hemolymph as the DNA template were done in solution. The optimized PCR conditions were as follows: primers (50 pmol each), dNTPs (200 µmole each), AmpliTaq Gold (Perkin-Elmer, Roche Molecular Systems, Inc., Branchburg, N.J.) polymerase (2.5 U/50 µl), $MgCl_2$ (2 mM), in PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3). Light mineral oil (50 µl) was overlaid on the top of the 50-µl reaction mixture to prevent evaporation and condensation during thermal cycling. A volume of 0.5 µl of template was used per 50 µl reaction volume. PCR was performed implementing the "hot start" method (Haff, 1993). The DNA template and all the reagents, including the AmpliTaq Gold polymerase, were combined and denatured at 94°C for 10 minutes in an

automatic thermocycler. The samples were then run for 25 cycles under the following conditions: denaturation at 94°C for 2 minutes; annealing at 55°C for 2 minutes; and polymerization at 72°C for 3 minutes. Following the last cycle, the samples were polymerized for 7 minutes at 72°C and then held at 4°C until they were analyzed.

Post-PCR Processing

Following the termination of PCR, the DNA solution was drawn off from beneath the mineral oil and placed into a clean 1.5-ml microfuge tube. Agarose gel electrophoresis was used to visualize the 1.6-kb amplified product. For agarose gel electrophoresis, 10 µl of the PCR reaction was analyzed on a 0.8% agarose gel, stained with ethidium bromide (0.5 mg/ml), and run in 0.5× TBE buffer (Maniatis et al., 1982). A 1-kb DNA ladder (Gibco BRL Life Technologies, Grand Island, N.Y.) and a DIG-labeled DNA molecular weight marker II (Roche Molecular Biochemicals, Indianapolis, Ind.) were used as markers. Two separate hybridization techniques were used to confirm the identity of the 1.6-kb amplified product. For Southern transfers, following electrophoretic separation, PCR reaction products were transferred onto positively charged nylon membranes (Roche Molecular Biochemicals). Dot blot hybridization analysis was performed by placing 1 µl of the PCR reaction product onto positively charged nylon membranes. For both Southern transfer and dot blot procedures, the 2-kb insert from clone BA401 was labeled using the Genius I Kit (Roche Molecular Biochemicals) according to the manufacturer's protocol for incorporation of DIG-11-dUTP by the random primed labeling reaction method. Hybridization with the probe was carried out overnight at 68°C using the DNA/DNA hybridization protocol described by Mari et al. (1998). Products were visualized using alkaline-phosphatase-labeled anti-DIG antibody and the reagents nitroblue tetrazolium and bromochloroindoyl phosphate.

Restriction Digests of PCR Amplified Products

Four restriction enzymes were identified from the IHHNV DNA sequence information (GenBank AF218266) that cut the amplified PCR product one or two times at locations along the length of the fragment. The enzymes, *Acc*I, *Kpn*I, *Nco*I, and *Nsi*I, were purchased from Roche Molecular Biochemicals and used according to the manufacturer's directions. The PCR products were amplified from the following isolates: *P. stylirostris* from Hawaii (1987), *P. stylirostris* from Ecuador (1992), *P. stylirostris* from Mexico (1996), and *P. monodon* from the Philippines (1996). The purified IHHNV (Mexico isolate, 1995) was used as the positive control. Prior to restriction enzyme analysis, the DNA from each 100-µl PCR reaction was precipitated using 1 µl of glycogen (20 mg/ml; Roche Molecular Biochemicals), 10 µl of 0.2 M EDTA, 11 µl of 4 M LiCl, and 366 µl of absolute ethanol. The precipitated DNA was resuspended in 25 µl of water, and 6 µl was used for each enzyme digestion. The digested fragments were visualized in 1.5% agarose gels (0.5 × TBE with 0.5 mg/ml ethidium bromide) using 1-kb and 100-bp DNA ladders (Gibco BRL) as markers.

Results

The PCR assay was optimized using purified virus as template, and these conditions were applied to hemolymph samples obtained from three different species of penaeid shrimp (*P. stylirostris, P. monodon,* and *P. vannamei*) and from four different geographic isolates of the virus (Mexico, Philippines, Hawaii, and Panama; Figure 1A). In all cases, the correct 1.6-kb fragment was observed after gel electrophoresis. Hemolymph from SPF shrimp and from shrimp infected with TSV or WSSV did not demonstrate any bands in the agarose gel. To confirm that the DNA fragments observed in the gel were specific to IHHNV, the bands in the gel were transferred to a nylon membrane and probed with the DIG-labeled probe from clone BA401 (Figure 1B). Only the samples known to contain IHHNV reacted with the IHHNV probe. Some bands larger than 1.6 kb were observed on the Southern blot that were not visible in the gel, but these were seen only in the samples that contained IHHNV. No DNA fragments were amplified in the uninfected SPF sample or in the samples containing TSV or WSSV as evidenced in both the gel picture and the Southern blot (Figure 1, 1A and B).

Two other geographic isolates of IHHNV, from Ecuador and Texas, were also confirmed by PCR amplification using tissue homogenates from shrimp with naturally acquired IHHNV infections (Figure 2A). The specificity was confirmed by dot blot hybridization of the amplified products using the IHHNV DIG-labeled probe (Figure 2B). Throughout the testing, no differences in the size of the amplified product or in the reactivity to the DIG-labeled probe were noted regardless of whether naturally or experimentally infected material was used.

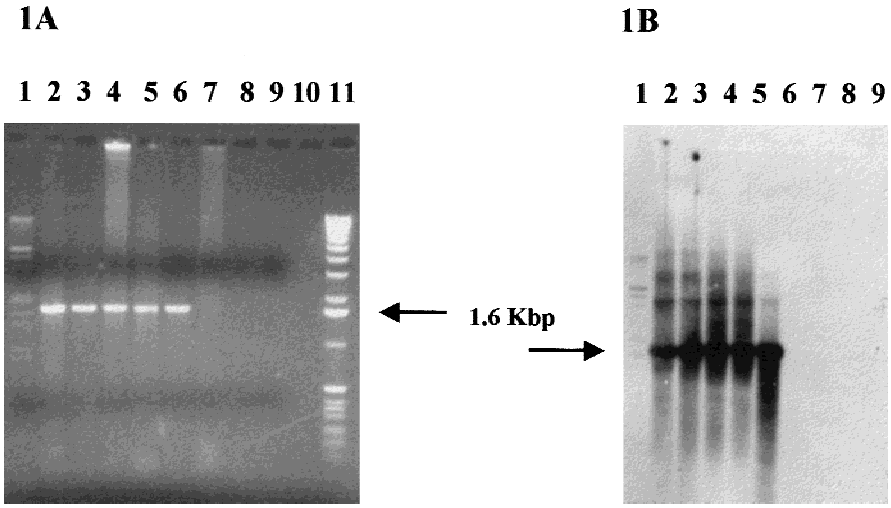

Figure 1. A: Electrophoresed 0.8% agarose gel of PCR amplification products of IHHNV-specific fragments using hemolymph as the DNA template from 3 different species of shrimp from 4 geographic areas. **B:** Southern blot hybridization of IHHNV DNA amplification products. Lane 1, DIG-labeled DNA marker; lane 2, *P. stylirostris* (Mexico, 1995); lane 3, *P. monodon* (Philippines, 1996); lane 4, *P. stylirostris* (Hawaii, 1997); lane 5, *P. vannamei* (Panama, 1997); lane 6, purified IHHNV positive control from *P. stylirostris* (Mexico, 1995); lane 7, SPF hemolymph negative control from *P. vannamei;* lane 8, TSV hemolymph; lane 9, WSSV hemolymph; lane 11, 1-kb DNA marker. The lanes on the blot correspond to the lanes in the agarose gel. The 1-kb DNA marker was not included in the hybridization.

Some hemolymph samples were tested in PCR after they had been boiled and the coagulated proteins had been removed. Using 0.5 µl of boiled hemolymph supernatant fluid as template in the PCR reaction resulted in more DNA being amplified than if 0.5 µl of unboiled hemolymph was used (Figure 3). Boiling the purified virus sample did not result in increased DNA amplification products (data not shown). Frozen homogenized tissue samples as well as pleopod samples preserved in 70% ethanol were also tested by PCR, and the amplified fragments were detectable in an agarose gel (Figures 2A and 3A) and by dot blot hybridization (Figures 2B and 3B).

A comparison was made between samples before and after PCR amplification using the dot blot method. In most cases, the dot blot reaction after amplification was significantly more intense than the reaction of the crude sample before amplification (Figures 2B, 3B, and 4B). However, some crude samples that elicited a strong dot blot reaction failed to react in the PCR test or reacted very weakly as detected by agarose gel or dot blot methods. When these crude samples were diluted 10^{-1} or 10^{-2} in water and then tested by the PCR method, they elicited strong reactions as visualized in agarose gels and dot blots (data not shown).

To determine the limits of sensitivity of the PCR assay for detection of IHHNV, plasmid DNA purified from the clone BA401 was diluted and tested by PCR. The results in Figure 4A demonstrate that a visible reaction product was evident in an agarose gel when plasmid DNA in the range of 500 ng/µl to 50 fg/µl was added to the 50-µl reaction mixture. The amplified products were also compared with the corresponding diluted plasmid DNA in a dot blot reaction (Figure 4B). The diluted plasmid DNA was detectable in the range of 500 ng to 5 pg. After PCR amplification, the dot blot reaction detected as little as 0.5 fg of starting plasmid DNA. The size of clone BA401 is 4598 bp; therefore, 0.5 fg of DNA is equivalent to 100 copies or units of the IHHNV insert that can be detected after PCR amplification when the products are visualized using dot blot hybridization.

Restriction lengths of the PCR products were compared using four of the isolates and the purified virus control. The isolates chosen were from naturally occurring infections spanning a time frame of almost a decade (1987–1996) and covering different geographic regions (Hawaii, Ecuador, Mexico, and the Philippines). The four enzymes used cut the 1.6-kb fragment one or two times, and restriction sites were located along the length of the amplified fragment. As shown in Figure 5, all of the isolates revealed identical restriction patterns, indicating that the PCR products obtained from the various isolates were homologous.

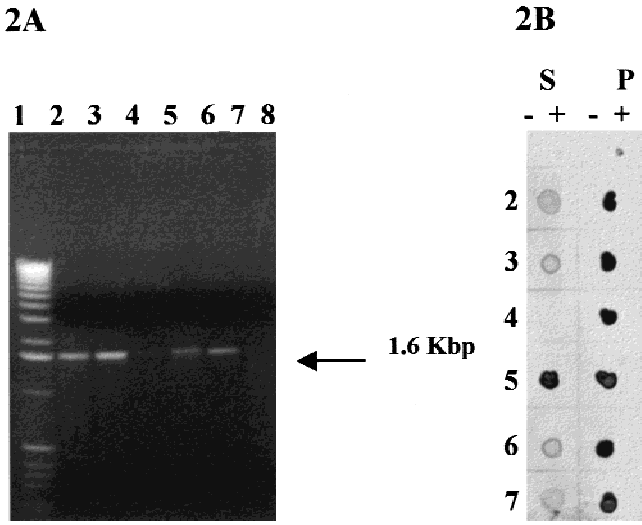

Figure 2. A: IHHNV-specific 1.6-kbp fragments visualized on a 0.8% agarose gel using homogenized tissue and hemolymph as the DNA template in PCR. Lane 1, 1-kbp DNA marker; lane 2, *P. stylirostris* tissue (Hawaii, 1987); lane 3, *P. stylirostris* tissue (Ecuador, 1992); lane 4, *P. vannamei* tissue (Texas, 1993); lane 5, *P. stylirostris* tissue (Mexico, 1996); lane 6, *P. stylirostris* hemolymph (Mexico, 1996); lane 7, *P. stylirostris* tissue (Philippines, 1996); lane 8: SPF hemolymph from *P. vannamei.* **B:** Dot blot hybridization using a DIG-labeled probe specific for IHHNV. The DNA templates prior to PCR amplification (S) and the corresponding amplified PCR products (P) are represented in the left and right columns, respectively. The negative SPF controls and the positive IHHNV controls are at the top of the dot blot. The numbers to the left of the blot indicate the sample lane from the agarose gel in A.

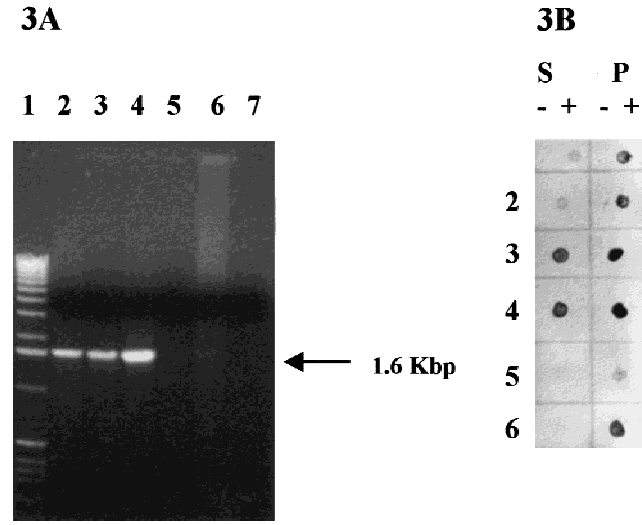

Figure 3. A: PCR amplification products electrophoresed in a 0.8% agarose gel using ethanol fixed tissue or hemolymph as the DNA template. Lane 1, 1-kb DNA marker; lane 2, *P. stylirostris* tissue fixed in ethanol (Hawaii, 1998); lane 3, *P. stylirostris* hemolymph, not boiled (Hawaii, 1998); lane 4, *P. stylirostris* hemolymph, boiled (Hawaii, 1998); lane 5, *P. vannamei* hemolymph, not boiled (Mexico, 1995); lane 6, *P. vannamei* hemolymph, boiled (Mexico, 1995); lane 7, SPF hemolymph from *P. vannamei.* **B:** Dot blot hybridization using a DIG-labeled probe specific for IHHNV. The DNA templates prior to PCR amplification (S) and the corresponding amplified PCR products (P) are represented in the left and right columns, respectively. The negative SPF controls and the positive IHHNV controls are at the top of the dot blot. The numbers to the left of the blot indicate the sample lane from the agarose gel in A.

Discussion

The results from this study indicate the usefulness and sensitivity of the PCR technique for detection of the penaeid shrimp virus IHHNV in frozen or ethanol-fixed tissues and in hemolymph samples. The primers chosen for DNA amplification were able to detect IHHNV obtained from several different geographic regions (Panama, Philippines, Mexico, Ecuador, Hawaii, and Texas) and in three different species of penaeid shrimp (*P. stylirostris, P. monodon,* and *P. vannamei*). In all cases, the expected 1.6-kb band was amplified as visualized in agarose gels (Figures 1A, 2A, and 3A). In addition, the PCR products from the different geographic regions and species of shrimp reacted with the DIG-labeled IHHNV probe in Southern blots (Figure 1B) and in dot blots (Figures 2B and 3B). The specificity of these primers for IHHNV was demonstrated by the absence of the 1.6-kb band in either gels or Southern blots when samples from uninfected SPF shrimp or from shrimp infected with TSV or WSSV were tested (Figure 1). Restriction fragment length analysis of the PCR products from four of the isolates were identical (Figure 5). The enzymes chosen for the analysis cut the fragment at a total of six different sites along the length of the amplified product (see Figure 5A).

Taken together, these results are significant because they indicate the nucleic acid similarity of IHHNV obtained from different geographic regions over a period of almost a decade and, hence, the stability of this particular parvovirus. The IHHNV genome (GenBank AF218266) is 4075 bases long; therefore, the 1681-base PCR product is approximately 40% of the viral genome. By amino acid sequencing of one of the IHHNV structural proteins and alignment with the translated DNA sequence, it was determined that the region encoded by the 1.6-kb amplified product encom-

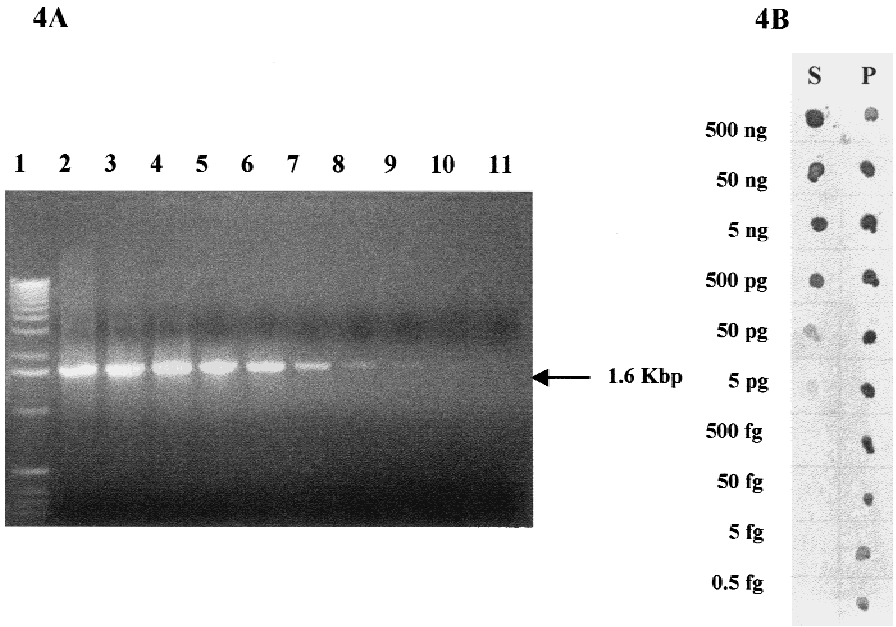

Figure 4. A: IHHNV-specific 1.6-kb fragments visualized on a 0.8% agarose gel using serial dilutions of plasmid DNA from clone BA401 as the DNA template in PCR. Lane 1, 1-kb DNA marker; lane 2, 500 ng; lane 3, 50 ng; lane 4, 5 ng; lane 5, 500 pg; lane 6, 50 pg; lane 7, 5 pg; lane 8, 500 fg; lane 9, 50 fg; lane 10, 5 fg; lane 11, 0.5 fg. **B:** Dot blot hybridization using a DIG-labeled probe specific for IHHNV. The serial dilutions of plasmid DNA from clone BA 401 prior to PCR amplification (S) and the corresponding samples after PCR amplification (P) are represented in the left and right columns, respectively. The numbers to the left of the blot indicate the concentration of plasmid DNA per microliter added to the PCR amplification mix.

passes the sequence for one of the major coat proteins of IHHNV (manuscript in preparation). Because of the small size of their DNA genome, there are evolutionary constraints on parvoviruses that result in few, if any, duplicated genes and little genetic drift (Strauss et al., 1990). In addition, presumably the small size of parvoviruses also limits their host range because they are dependent on the host for many of their functions (Kurstak et al., 1977). The results presented here demonstrate that a significant region of the IHHNV genome is highly homologous among isolates obtained from different geographic regions over a period of almost a decade. The stability of the nucleic acid sequence in the region of the coat protein indicates that IHHNV is a highly stable virus, which undergoes minimal spontaneous mutation in its natural host species.

Most PCR protocols recommend extraction of the nucleic acid from the sample prior to amplification. This purification step concentrates the DNA and removes any polymerase inhibitors that may be inherent in a biological sample, but increases both the cost and the time required to perform the assay. This study sought to develop a rapid, simple, and sensitive test for the presence of the virus in shrimp tissues and hemolymph; therefore, crude samples were chosen for use as the template. Inhibitors to PCR have been reported in homogenates of shrimp tissue (Wang et al., 1996). However, these investigators looked primarily at homogenates of hepatopancreas, an organ that contains numerous enzymes, polyphenolic compounds, and pigments, some of which may inhibit DNA polymerase activity or bind to divalent cations. In the present study, neither the tissue homogenates prepared from pleopods or the gnathothorax (with the carapace and hepatopancreas removed) nor the hemolymph inhibited the PCR assay under the conditions described (Figures 1, 2, and 3).

Although the data are not presented, the use of sodium citrate as an anticoagulant in hemolymph was capable of inhibiting the PCR assay if used in excessive amounts, presumably by irreversibly binding necessary magnesium ions in the reaction mixture. This problem was eliminated by using no more than 10 µl of a 10% solution per 300 µl of hemolymph drawn. Without the addition of the anticoagulant, the hemolymph rapidly forms a clot. This could still be used as sample for PCR if the clotted hemolymph was centrifuged briefly and the small amount of extruded liquid was used for the assay. Likewise, hemolymph samples that were previously boiled to denature the viral strands prior to the dot blot assay elicited stronger reactions in the PCR assay than the unboiled hemolymph (Figure 2). In this instance, the boiled hemolymph was centrifuged to pellet the large amount of coagulated protein and the clear liquid remaining on top was used for PCR. The boiling step may cause the release of virions from hemocytes or serve to concentrate the virus in the small amount of liquid remaining after the proteins coagulate. In any case, if necessary, samples of

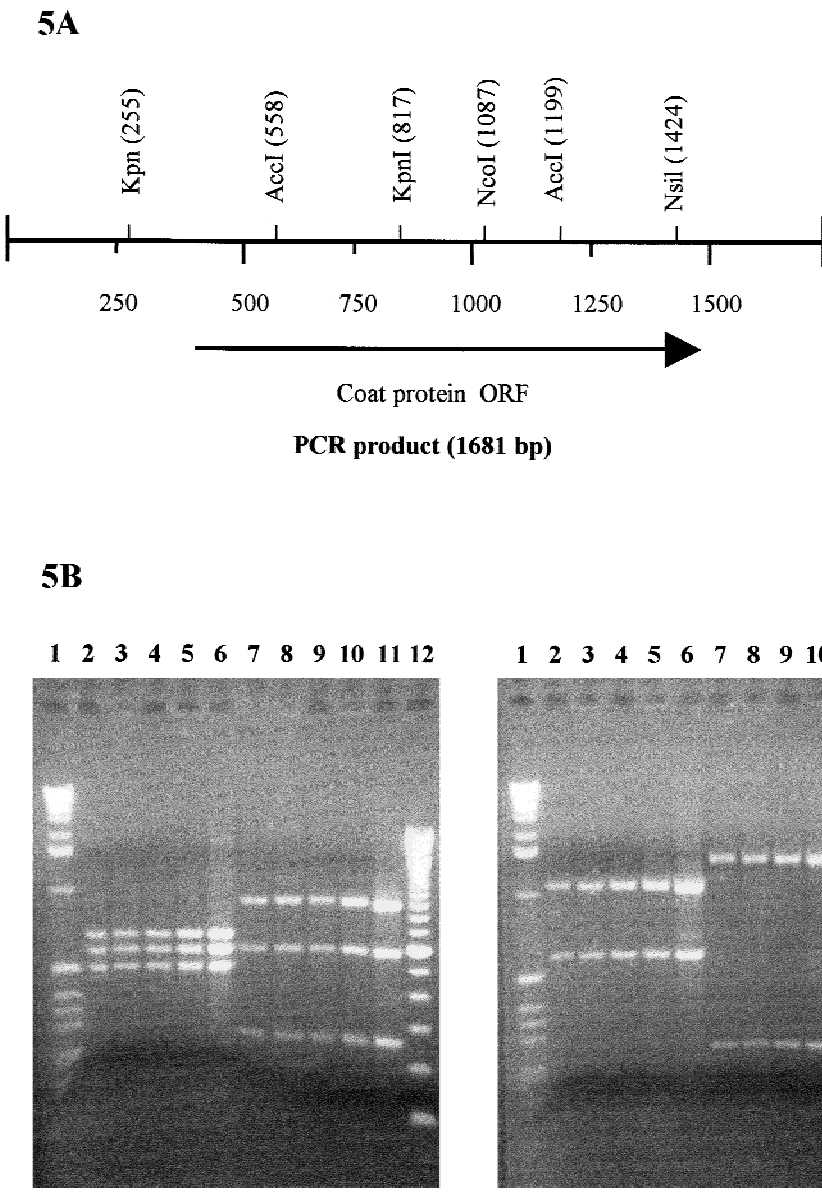

Figure 5. A: Schematic drawing of the location of the restriction sites for *Acc*I, *Kpn*I, *Nco*I, and *Nsi*I along the 1.6-kb PCR product of IHHNV. The position of the open reading frame (ORF), which codes for the viral coat protein, is also indicated. **B:** Restriction digest patterns of the PCR product from four different isolates of IHHNV. Lane 1, 1-kb DNA marker; lane 12, 100-bp DNA marker; lanes 2–6 (left gel), *Acc*I digestions for *P.s.* HI (1987), *P.s.* Ec (1992), *P.s.* Mx (1996), *P.m.* Ph (1996), and positive control (*P.s.* Mx 1995), respectively; lanes 7–11 (left gel), *Kpn*I digestions for *P.s.* HI (1987), *P.s.* Ec (1992), *P.s.* Mx (1996), *P.m.* Ph (1996), and positive control (*P.s.* Mx 1995), respectively; lanes 2–6 (right gel), *Nco*I digestions for *P.s.* HI (1987), *P.s.* Ec (1992), *P.s.* Mx (1996), *P.m.* Ph (1996), and positive control (*P.s.* Mx 1995), respectively; lanes 7–11 (right gel), *Nsi*I digestions for *P.s.* HI (1987), *P.s.* Ec (1992), *P.s.* Mx (1996), *P.m.* Ph (1996), and positive control (*P.s.* Mx 1995), respectively. *P.s.* indicates *Penaeus stylirostris, P.m., P. monodon;* HI, Hawaii; Ec, Ecuador; Mx, Mexico; Ph, Philippines.

boiled hemolymph can be tested first in a dot blot test, then negative or weakly positive samples can be confirmed using the more sensitive PCR assay.

Some crude samples, which gave a strong reaction in the dot blot assay, were weak or negative by PCR unless the samples were diluted by a factor of 10 to 100. Possible explanations for false-negative reactions may be the presence of an inhibitor that can be diluted out or of too much template in the PCR mix so that efficient amplification of the DNA does not occur. In this regard, purified plasmid DNA from clone BA401 elicited the 1.6-kb band visible in agarose gels in the range of 50 fg to 500 ng added per 50-µl reaction and in dot blots in the range of at least 0.5 fg to 500 ng added per 50-µl reaction (Figure 4).

The thermal cycling parameters used in this study for IHHNV PCR were developed using tissue homogenates and hemolymph as the source of viral template. The most important aspect of the cycling procedure was the incorporation of the "hot start" method at the beginning of the thermal cycling. This technique enhances the specific product amplification through polymerase activity and helps eliminate nonspecific products and primer-dimer formation (Haff, 1993). In the past, the drawback to using this method was the need to reopen the reaction tubes to add the *Taq* polymerase after the initial denaturation and annealing step, which was inconvenient and increased the risk of contamination (Chou et al., 1992). The use of AmpliTaq Gold, which is inactive until denaturation has occurred, permits

the addition of enzyme at the beginning of the thermal cycling.

The advantages of PCR for detection of IHHNV over other DNA-based detection methods, such as in situ hybridization, are simplicity, speed, and the nondestructive methods of sample acquisition. Unlike previously reported methods for identifying viral infections in penaeid shrimp (Lo et al., 1996; Chang et al., 1998), the technique presented here obviates the need for virus purification or DNA extraction. The PCR methods described are simpler and faster than standard in situ detection methods, which require Davidson's AFA or formalin-fixed samples that are embedded in paraffin, sectioned, and then processed. The results from the PCR techniques developed in this study can be visualized on agarose gels within 24 hours of processing the study sample or within 48 hours using dot blot hybridization. Another clear advantage of PCR over other currently available assay methods for IHHNV is sensitivity. PCR permits the detection of most viral pathogens that can be difficult to identify with conventional culturing techniques. Because there are no methods by which to grow shrimp viruses in culture and no known specific immune products made by shrimp in response to a virus that can be measured, a PCR approach can yield the greatest sensitivity for detection of IHHNV.

Though dot blot techniques can determine samples positive for IHHNV, PCR techniques increase the likelihood that low-level infections will be detected in the amplified product. As can be seen in the dot blots in Figures 2B and 3B, the colorimetric reactions of some of the shrimp homogenate samples that were positive for IHHNV were faint to barely perceptible. This type of reaction could be difficult to distinguish from a background reaction. When the PCR-amplified product is compared with that of the homogenate, there is no doubt that the sample contained IHHNV.

Another important consideration to be drawn from this study is the incorporation of PCR into a screening program for SPF shrimp broodstock. Hemolymph can be drawn repeatedly from the same animal over an extended period of time, without damaging the animal. A combination of broodstock screening and random sampling of postlarval samples, using the PCR techniques developed in this study, provides a highly sensitive and rapid method for the detection of IHHNV in individual farm-raised or wild shrimp specimens or in populations.

In conclusion, the results of this study indicate that IHHNV can be detected in shrimp tissue homogenate and hemolymph through the use of PCR. Low levels of the virus, which may not have been detectable by previously existing assay methods, can be determined conclusively through amplification of the viral DNA by PCR.

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References

Bell, T.A., and Lightner, D.V. (1988). *A Handbook of Normal Penaeid Shrimp Histology.* Baton Rouge, La.: World Aquaculture Society.

Bonami, J.R., Trumper, B., Mari, J., Brehelin, M, and Lightner, D.V. (1990). Purification and characterization of the infectious hypodermal and haematopoietic necrosis virus of penaeid shrimps. *J Gen Virol* 71:2657–2664.

Chang, P.S., Tasi, D.H., and Wang, Y.C. (1998). Development and evaluation of a dot blot analysis for the detection of white spot syndrome baculovirus (WSBV) in *Penaeus monodon. Fish Pathol* 33:45–52.

Chou, Q., Russell, M., Birch, D.E., Raymond, J., and Bloch, W. (1992). Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications. *Nucleic Acids Res* 20:1717–1723.

Haff, L. (1993). PCR: applications and alternative technologies. *Biotechnology* 11:938–939.

Kalagayan, G., Godin, D., Kanna, R., Hagino, G., Sweeney, J., Wyban, J., and Brock, J. (1991). IHHN virus as an etiological factor in runt-deformity syndrome of juvenile *Penaeus vannamei* cultured in Hawaii. *J World Aquaculture Soc* 22:235–243.

Kurstak, E., Tijssen, P., and Garzon, S. (1977). Densonucleosis viruses (Parvoviridae). In: *The Atlas of Insect and Plant Viruses,* Maramorosch, K. (ed). New York, N.Y.: Academic Press, 67–77.

Lightner, D.V. (1996). *A Handbook of Shrimp Pathology and Diagnostic Procedures for Diseases of Cultured Penaeid Shrimp.* Baton Rouge, La.: The World Aquaculture Society.

Lightner, D.V., Redman, R.M., Bell, T.A., and Brock, J.A. (1983a).

Detection of IHHN virus in *Penaeus stylirostris* and *P. vannamei* imported into Hawaii. *J World Maricul Soc* 14:212–225.

Lightner, D.V., Redman, R.M., and Bell, T.A. (1983b). Infectious hypodermal and hematopoietic necrosis, a newly recognized virus disease of penaeid shrimp. *J Invertebr Pathol* 42:62–70.

Lightner, D.V., Bell, T.A., Redman, R.M., Mohney, L.L., Natividad, J.M., Rukyani, A., and Poernomo, A. (1992a). A review of some major diseases of economic significance in penaeid prawns/shrimps of the Americas and Indopacific. In: *Diseases in Asian Aquaculture, Fish Health Section,* Shariff, I.M., Subasinghe, R.P., and Arthur, J.R. (eds). Manila, Philippines: Asian Fisheries Society, 57–80.

Lightner, D.V., Williams, R.R., Bell, T.A., Redman, R.M., and Perez, A.L.A. (1992b). A collection of case histories documenting the introduction and spread of the virus disease IHHN in penaeid shrimp culture facilities in Northwestern Mexico. *ICES Mar Sci Symp* 194:97–105.

Lightner, D.V., Redman, R.M., Poulos, B.T., Nunan, L.M., Mari, J.L., and Hasson, K.W. (1997). Risk of spread of penaeid shrimp viruses in the Americas by the international movement of live and frozen shrimp. *Rev Sci Tech Off Int Epiz* 16:146–160.

Lo, C.F., Leu, J.H., Ho, C.H., Chen, C.H., Peng, S.E., Chen, Y.T., Chou, C.M., Yeh, P.Y., Huang, C.J., Chou, H.Y., Wang, C.H., and Kou, G.H. (1996). Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction. *Dis Aquat Org* 25:133–141.

Lotz, J.M., Browdy, C.L., Carr, W.H., Frelier, P.F., and Lightner, D.V. (1995). USMSFP suggested procedures and guidelines for assuring the specific pathogen status of shrimp broodstock and seed. In: *Swimming Through Troubled Waters! Proceedings of the Special Session on Shrimp Farming,* Browdy, C.L., and Hopkins, J.S. (eds). Baton Rouge, La.: World Aquaculture Society, 66–75.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory.

Mari, J., Bonami, J.R., and Lightner, D.V. (1993). Partial cloning of the genome of infectious hypodermal and hematopoietic necrosis virus, an unusual parvovirus pathogenic for penaeid shrimps; diagnosis of the disease using a specific probe. *J Gen Virol* 74:2637–2643.

Mari, J., Bonami, J.R., and Lightner, D.V. (1998). Taura syndrome of penaeid shrimp: cloning of viral genome fragments and development of specific gene probes. *Dis Aquat Org* 33:11–17.

Morales-Covarrubias, M.S., Nunan, L.M., Lightner, D.V., Mota-Urbina, J.C., Garza-Aguirre, M.C., and Chavez-Sanchez, M.C. (1999). Prevalence of IHHNV in wild broodstock of *Penaeus stylirostris* from the upper Gulf of California, Mexico. *J Aquat Anim Health* 11:296–301.

Mullis, K.B., and Faloona, F.A. (1987). Specific synthesis of DNA in vitro via a polymerase-catalysed chain reaction. *Methods Enzymol* 155:335–350.

Pantoja, C.R., Lightner, D.V., and Holtschmit, K.H., (1999). Prevalence and geographic distribution of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in wild blue shrimp *Penaeus stylirostris* from the Gulf of California, Mexico. *J Aquat Anim Health* 11:23–34.

Strauss, E.G., Strauss, J.H., and Levine, A.J. (1990). Virus evolution. In: Raven Press, Fields, B.N., and Knipe, D.M. (eds). New York, N.Y.: *Fields Virology,* 2nd ed., 167–190.

Wang, S.Y., Hong, C., and Lotz, J.M. (1996). Development of a PCR procedure for the detection of *Baculovirus penaei* in shrimp. *Dis Aquat Org* 25:123–131.