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In situ Hybridization of White Spot Disease Virus in Experimentally Infected Penaeid Shrimp

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Abstract White spot disease (WSD), resulting in more than 90% mortality of aquacultured penaeid shrimp, has been reported off the southern and western coasts of Korea since 1993. The pathogen of WSD has been identified as being a virion with an envelope around a central nucleocapsid, and with an average size of 167 nm in diameter and 375 nm in length. In the present study, an *in situ* hybridization technique was developed as a rapid, sensitive, and specific diagnostic assay for the WSD virus infection in shrimp. Furthermore, the pathological changes of WSD, in shrimp experimentally infected with WSD viruses, were investigated. Using a biotinylated 643 bp probe obtained from a PCR using primers specific to the rod-shaped virus of *Penaeus japonicus* (RV-PJ), positive signals were detected in both naturally and experimentally infected shrimps. The *in situ* hybridization revealed positive reactions in the nuclei of the stromal matrix cells in the lymphoid organ, epithelia of the gills, foregut, epidermis, and hematopoietic cells of the interstitial tissues, suggesting the presence of WSD virus. This result indicates that the *in situ* hybridization method can be useful for a rapid and sensitive detection of WSD viruses in shrimp.

Key words: *In situ* hybridization, white spot disease virus, shrimp

Since 1993, the sudden and massive deaths of penaeid shrimps have occurred in intensive shrimp aquaculture nurseries off the southern and western coasts of Korea [8]. This disease attacking shrimps has been named white spot disease (WSD), as it is characterized by 2–6 mm sized-white spots on the inside of the shrimp carapace along with reddish discoloration [8, 9]. Both morbidity and mortality of the disease are approaching 100%. Light microscopic examination showed a massive necrosis of the lymphoid organ, and degeneration and necrosis of the epithelia of the epidermis and foregut [8, 9, 13]. Amphophilic or basophilic intranuclear inclusion bodies were also readily found in the epithelia of the epidermis, foregut, and gills [9]. A similar disease has also occurred in Japan, Thailand, and Taiwan since the early spring of 1993 [1-4, 14]. The causative agents for WSD have been identified as viruses or a group of closely related viruses, including the rod-shaped virus of *Penaeus japonicus* (RV-PJ) in Japan, the systemic ectodermal and mesodermal baculovirus (SEMBV) in Thailand, and the white spot baculovirus (WSBV) in Taiwan [7]. There is a slight difference between the particle size and PCR data of these viruses, however, they are taxonomically all baculoviridae [3, 7, 10, 14]. However, there was a recent report that WSD virus might be a representative of a new virus family, Whispoviridae, because the major viral protein of WSD virus showed homology to the baculovirus structure protein [12].

The infection of shrimp by baculoviruses has caused great financial loss to the shrimp aquaculture industry because there is currently no effective measure for controlling WSD viruses. As a result, avoidance is the only available control method. Several methods including nucleic acid- and/or immunologically based detection protocols have been described for the detection of the infectious WSD [5-7, 11]. However, it is still necessary to develop a rapid, sensitive, and accurate diagnostic assay for the detection of the WSD virus.

In order to develop a diagnostic assay in the present study, the pathological changes of WSD, in shrimp experimentally infected with WSD viruses, were investigated. An *in situ* hybridization method was found to be very rapid and sensitive for detecting the presence of a WSD virus in several organs of shrimp experimentally infected with this virus.

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MATERIALS AND METHODS

Shrimp

Shrimps were reared in aquariums. The aquariums were layered with 5 cm of sea sand at the bottom, filled with 15 l of seawater, and equipped with an oxygen supplier. Shrimps were fed 10 mm pellet feed manufactured by Shinchun Feed Co. (Incheon, Korea). The experiment was conducted at 25–28°C.

Virus

Shrimps that had been naturally infected with the baculovirus were collected from a culture farm at Taean in Choongchung Province located on the western coast of Korea and stored at -20°C until use. Epidermis, lymphoid organ, and foregut from the shrimp were homogenized in 10 volumes of phosphate buffered saline (PBS). The homogenate was centrifuged at 3,000 rpm at 5°C for 5 min, and the supernatant was then filtered through a 0.45-μm syringe filter. The filtrate was intramuscularly inoculated into ten healthy shrimps. The filtrate was prepared again from shrimps that had died from the experimental infection and then used as the virus inoculum. This inoculum was injected into the abdominal muscle of fifteen healthy shrimps. Five healthy shrimps were reared in another aquarium and used as the negative control.

Light Microscopic Examination

The shrimps were sacrificed at 18, 36, and 54 h post inoculation (PI) and fixed in 10% neutral buffered formalin for one week. After fixation, the cephalothorax was cut into 5-mm thickness from the base of the rostrum to the end of the cephalothorax. The tissues were dehydrated in a series of graded alcohol and then embedded in paraffin wax. The embedded tissue samples were cut into 3–5 μm sections and stained with haematoxylin and eosin (H&E).

Transmission Electron Microscope

The lymphoid organ and epidermis of the sacrificed shrimp were fixed in 2.5% glutaldehyde in a cacodylate buffer (pH 7.2) at 4°C for 4–5 h, and then post-fixed in 1% osmium tetroxide in the cacodylate buffer (pH 7.2) for 100 min. The tissue was then dehydrated and embedded in Epoxy 812. A 70-nm thick ultrathin section was made using a diamond knife, and these sections were then stained with uranyl acetate and lead citrate, and examined using a JEM-100CX II (JEOL) electron microscope.

Virus Purification and Extraction of Viral DNA

For the preparation of a DNA template, purification of the virus and extraction of the viral DNA were carried out as described previously elsewhere [8]. Briefly, shrimps were rinsed with a cold TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.6). The cephalothoraxes were soaked in a cold extraction buffer (20 mM HEPES, 0.4 N NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 2.5 mM phenylmethylsulfonylfluoride, 1 μg/ml leupeptin, 1.6 μg/ml pepstatin, 2 μg/ml aprotinin, and 1 μg/ml bestatin) and homogenized by a bead beater (20 cycles of 20 sec pulse and 20 sec rest). DNase (1 μg/ml) and RNase (1 μg/ml) were added to the homogenate and the mixtures was stirred slowly using a glass rod at room temperature. After 30 min, NaCl (final conc. 1 M) was added to the homogenate that was then kept in ice water for 1 h. The homogenate was centrifuged at 1,000 ×g for 10 min. The resulting supernatant was incubated in a TE buffer containing 0.5% (W/V) SDS, 1 mM EDTA, and 1 mg/ml proteinase K at 65°C for 2 h and extracted with phenol-chloroform, followed by ethanol precipitation.

Amplification of Fragment using PCR

The first primer of RV-PJ in the sense orientation (5'-GACAGAGATATGCACGCCAA-3') and second primer in the antisense orientation (5'-ACCAGTGTTTCGTCAT-GGAG-3'), as designed by Takahashi *et al.* [10], were used to amplify a 643 bp fragment using PCR. The reaction mixture contained 10 ng template DNA in a 100 μl reaction mixture containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 100 pmol each of primers, 200 μM deoxynucleotide triphosphate, and 2.5 units of *Taq* DNA Polymerase (Takara *Taq*, Takara, Tokyo). The mixture was incubated for 30 cycles in a PTC-100™ Programmable Thermal Controller (MJ Research, Inc.) for 0.5 min at 95°C, 1 min at 58°C, and 1 min at 72°C, followed by an extension for 5 min at 72°C after the last cycle. The resulting product was analyzed by 0.8% agarose gel electrophoresis. The band was cut with a razor blade and the DNA was extracted from the gel slice using a Geneclean II kit (Bio101, CA, U.S.A.).

Biotin-Labeling of Probe

Biotin labeling was performed using a Biotin-Chem-Link kit (Boehringer Mannheim, Germany). A reaction mixture was made up with a total volume of 20 μl containing 1 μg of DNA mixed with 1 μl of Biotin-Chem-Link vial 1, and the final volume was adjusted to 20 μl with sterile Millipore filtered water. The reaction mixture was incubated at 98°C for 30 min and briefly centrifuged to collect precipitate. The reaction was stopped by the addition of 5 μl stopping solution (Biotin-Chem Link vial 2).

In situ Hybridization

The formalin-fixed and paraffin wax-embedded tissues were cut into 5 μm sections, mounted on "superfrost/plus" slides (Fisher Scientific, U.S.A.), and stored at room temperature. Just prior to use, the sections were baked at 60°C for 20 min, dewaxed in xylene, and rehydrated in a series of graded ethanol for 5 min. Then, the slides were dipped into 1%

skimmed milk in PBS for 20 min to block any nonspecific responses to proteins and then washed with PBS. For deproteinization, the sections were treated with 0.2 N HCl for 20 min and digested with 50 μg/ml proteinase K (Sigma, U.S.A.) in PBS (pH 7.4, 0.01 M) at 36°C for 20 min, followed by fixation with 4% paraformaldehyde in PBS for 10 min. After rinsing twice with PBS, the sections were acetylated in 300 ml of 0.1 mM triethanolamine-HCl buffer (pH 8.0) with 0.75 ml acetic anhydride (0.25%). After standing for 5 min, an additional 0.75 ml of acetic anhydride was added, and the slides were rinsed in 2× SSC (saline sodium citrate) 5 min later. In a pre-hybridization step, the sections were covered at 45°C for 60 min with standard hybridization buffer that consisted of 20× SSC containing 50% deionized formamide, 50× Denhart's solution, Salmon sperm DNA, 10% SDS, and 50% dextran sulfate. Biotin-labeled probe (5 ng/ml) diluted in the standard hybridization buffer was denatured by boiling for 10 min and quenched on ice before use. Fifty-μl of the denatured biotin-labeled probe was placed over the section to eliminate any evaporative loss from the probe during the *in situ* hybridization, and the slides were then sealed by Easiseal (Hybaid, U.K.). For denaturing the target DNA in the tissue sections, each slide was heated at 95°C for 5 min and hybridized overnight at 45°C using an *in situ* PCR machine (Touchdown, Hybaid, U.K.). The following day, the slides were thoroughly washed, twice with 4× SSC at room temperature for 5 min, then twice each with 2× SSC at 37°C for 10 min, and finally twice with 2× SSC and 0.2× SSC at room temperature for 5 min. The biotin-labeled probes were detected using an ABC kit (Vectastatin. U.S.A.) and a DAB kit (Vector Laboratories, U.S.A.). Negative control sections were prepared from healthy shrimps that had never been exposed to the WSD virus.

RESULTS

Clinical Signs

All ten shrimps, inoculated with the filtrate which was prepared from the shrimps naturally infected with the baculovirus, died within 72-h post-inoculation (pi). The experimentally infected shrimps with the passaged virus started to show clinical signs from 20-h pi, and all fifteen shrimps died within 50-h pi. Typically, the clinical signs included a characteristic S-shape swimming pattern, lethargy, anorexia, opaque musculature, and a laterally recumbent posture. The shrimp with WSD did not respond to touch. At necropsy, characteristic white spots inside the carapace and red coloration of the body were observed.

Histopathological Findings

The most pronounced histopathological changes were found in the lymphoid organ, epithelia of the epidermis, foregut, and gills. In the lymphoid organ, the stromal matrix cells and epithelia of the tubule were massively necrotized, resulting in the destruction of the tubular structure (Fig. 1). In addition, the epithelia of the epidermis were either degenerated or necrotic. Many amphophilic or basophilic nuclear inclusion bodies were observed in the nuclei of the degenerating and necrotic epithelial cells of the epidermis and stromal matrix of the lymphoid organs. A nucleus containing an inclusion body had a space between the nuclear membrane and the inclusion body. Intranuclear inclusion bodies were also observed in the epithelia of the foregut and gills. There were no degenerating or necrotic cells in the epithelium of the hepatopancreas. However, some of the interstitial cells of the hepatopancreas did include amphophilic intranuclear inclusion bodies.

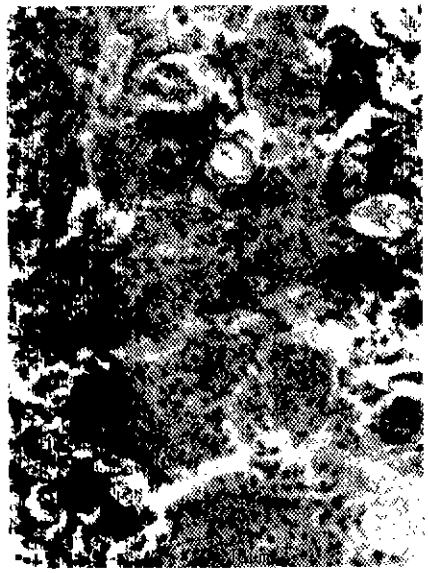

Fig. 1. Histological section of lymphoid organ from penaeid shrimp experimentally infected with WSDV.
A massive necrosis of the stromal matrix cells and destruction of the tubular structure were observed. Circular basophilic nuclear inclusion bodies were observed in the stromal matrix cells by haematoxylin eosin stainings

Transmission Electron Microscopic Findings

Rod-shaped virions were found mostly in the nucleus of the stromal matrix cells of the infected lymphoid organs and epithelial cells of the epidermis obtained from the experimentally infected shrimps. Furthermore, the inclusion bodies that appeared in the degenerating and necrotic cells were identified as clusters of virus particles. These virions were composed of an envelop around a central nucleocapsid. The average size of a complete virion was 167 nm in diameter and 375 nm in length, and the size of a nucleocapsid was 75 nm in diameter and 290 nm in length (Fig. 2). A few virus particles were also seen in the cytoplasm. Additionally, mitochondria in the infected cells

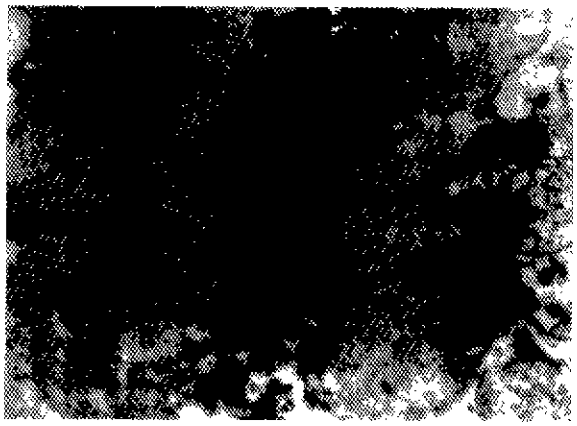

Fig. 2. Electron micrograph of epithelia of epidermis obtained from penaeid shrimp experimentally infected with WSDV.
Rod-shaped virions were found mostly in the nucleus of the epithelia In addition, a few virus particles were also seen in the cytoplasm (×8,000).

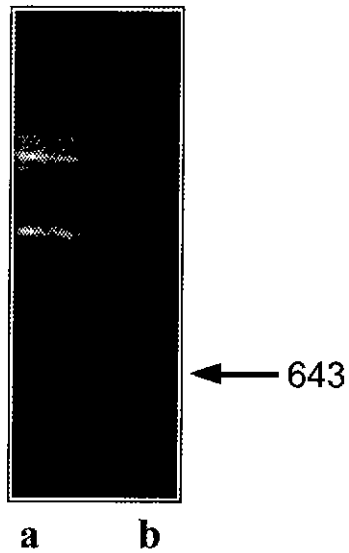

Fig. 3. Ethidium bromide-stained agarose gel of PCR-amplified fragments.
Primers specific for RV-PJ were used for the reaction using a DNA template prepared from the experimentally infected shrimp (lane b). Lane a: DNA size markers.

of the lymphoid organ and epithelial cells of the epidermis were swollen, whereas the rest of the cellular organelles were destroyed.

PCR

Amplification of the expected 643 bp fragment was evident in all the spontaneously and experimentally infected shrimps (Fig. 3). No amplification product at 643 bp was present in the healthy control shrimps.

In situ Hybridization

The sections of tissue samples from the spontaneously and experimentally infected shrimps reacted with the biotin-labeled probe upon *in situ* hybridization. Positive signals were detected in the nuclei of various tissues originating from the meso- and ectoderm of the diseased shrimp, including the lymphoid organ, foregut, epithelium, and gills (Figs. 4a, 4b, 4c). In most of the nuclei of the epidermis along the inside of the foregut, strong positive signals were observed. Only a small number of positive signals were seen in the hematopoietic cells in the interstitial cells. A small numbers of positive signals were observed in the epidermis of the gill and the epithelia of the epidermis in the foregut. The tissue sections from the control shrimp showed no hybridization signals (Fig. 5).

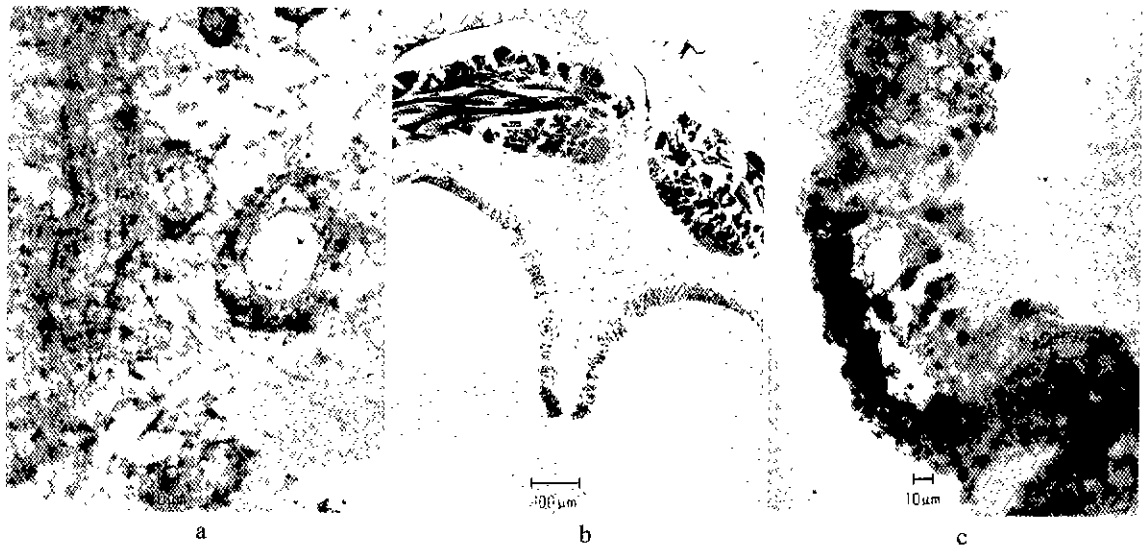

Fig. 4. *In situ* hybridization with the biotin-labeled DNA probe for the virus.
The dark precipitate shows that the probe reacted strongly with the intranuclear inclusion bodies containing WSDV (a) Lymphoid organs, (b) Foregut, (c) Epithelium.

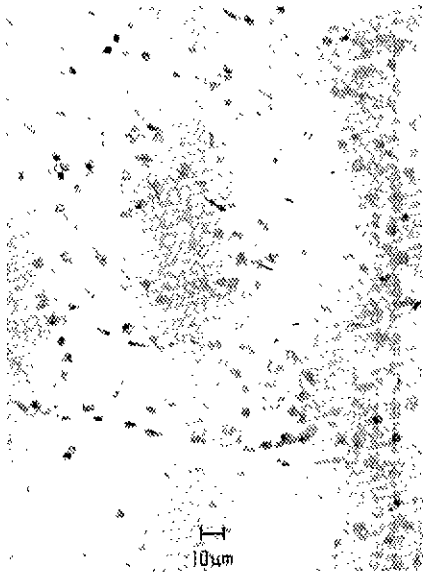

Fig. 5. Section of lymphoid organ from normal penaeid shrimp after *in situ* hybridization with the biotin-labeled DNA probe for the virus.

DISCUSSION

Until now, over a dozen viruses have been identified in penaeid shrimp, including five major viral families - Parvoviridae, Baculoviridae, Reoviridae, Rhabdoviridae, and Togaviridae [13]. Among these viruses, baculoviruses have been found to be the most common pathogens and are widely distributed in cultured shrimps [13]. Since 1993, a massive mortality has occurred in penaeid shrimps, *Penaeus orientalis,* cultured in Korea. The characteristic gross findings in the infected shrimps are the appearance of numerous white spots on the inside of the carapace accompanied by a pale body and reddish discoloration [8, 9]. In this study, many nuclei with amphophilic to basophilic inclusion bodies were observed in the various meso- and ectodermal tissues, such as the epithelia of the epidermis, foregut, gills, and lymphoid organ. Based on its morphological characteristics, the virus was found to be similar to WSDV in Taiwan [13]. The WSDV is spindle- or rod-shaped, is 70-150 × 250-380 nm in size, and has double-stranded DNA of 150 kbp [3]. However, PCR with primers specific to RV-PJ gave a PCR product with the expected size of 643 bp, whereas PCR primers specific to WSDV gave no product [3, 10]. When the nucleotide sequence of the 643 bp PCR product was determined, it showed 100% homology to the one reported by Takahahi *et al.* [10]. Thus, the WSDV in infected Korean shrimp has some characteristics of RV-PJ and some of WSDV found in Taiwan [3, 9, 10].

In this study, the experimentally infected shrimp showed the same clinical signs and histopathological findings as those found in the naturally infected shrimps. Using TEM observation, the presence of the baculovirus was established in the lesions. The organ that showed the most pronounced histopathological changes was the lymphoid organ. The immature viruses with capsid originators, empty capsids, nucleocapsids, and circular envelopes were observed in the nucleoplasm, suggesting that virogenesis was taking place in the nucleus [13].

Since WSDVs spread very fast and kill shrimps in less than 3-4 days, early detection and prevention of wide spread of the viral diseases are very important in the shrimp industry, because there is no treatment for virus infection. These days, various techniques have been developed for the detection of WSDV. Using an *in situ* hybridization and PCR in this study, viral DNA was detected in both the naturally and experimentally infected shrimps. Although the intensity of the signal was different in each slide, the dark brown precipitates to indicate a positive reaction to the DNA sequence of RV-PJ coincided with the inclusion bodies of the infected cells. These findings indicate that the results of the *in situ* hybridization were consistent with those obtained from the histopathological observations [8, 9]. A comparison with H&E stained sections from the same tissue samples indicated that most of the positive cells were distributed among the stromal matrix cells of the lymphoid organ and epithelia of the epidermis. Accordingly, it can be concluded that the *in situ* hybridization method is useful for the rapid and sensitive detection of WSD viruses in shrimp.

Acknowledgments

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REFERENCES

1. Asian Shrimp Culture Council. 1995. Mass mortality caused by systemic bacilliform virus in cultured penaeid shrimp, *Penaeus monodon*, in Thailand. *Asian Shrimp News* **21:** 2-4.
2. Inouye, K., S. Miwa, N. Oseko, H. Nakano, T. Kimura, K. Momoyama, and M. Hiraoka. 1994. Mass mortality of cultured kuruma shrimp *Penaeus japonicus* in Japan in 1993: Electron microscopic evidence of the causative virus. *Fish Pathol.* **29:** 149-158.
3. Lo, C. F., J. H. Leu, C. H. Ho, C. H. Chen, S. E. Peng, Y. T. Chen, C. M. Chou, P. Y. Yeh, S. J. Huang, H. Y. Chou, C. H. Wang, and G. H. Kou. 1996. Detection of baculovirus associated with white spot syndrome (WSS) in penaeid shrimp using polymerase chain reaction. *Dis. Aquatic Organisms* **25:** 133-141.
4. Momoyama, K., M. Hiraoka, K. Inouye, T. Kimura, and H. Nakano. 1995. Diagnostic techniques of the rod-shaped

nuclear virus infection in kuruma shrimp. *Penaeus japonicus*. *Fish Pathol.* **30:** 263–269.

5. Nadala, E. C. and P. C. Loh. 2000. Dot-blot nitrocellulose enzyme immunoassays for the detection of white-spot virus and yellow-head virus of panaeid shrimp. *J. Virol. Methods* **84:** 175–179.
6. Nadala, E. C., L. M. Tapay, S. Cao, and P. C. Loh. 1997. Detection of yellow virus and chinese baculovirus in panaeid shrimp by the western blot technique. *J. Virol. Methods* **69:** 39–44.
7. Nunan, L. M. and D. V. Lightner. 1997. Development of non-radioactive gene probe by PCR for detection of white spot syndrome virus (WSSV). *J. Virol. Methods* **63:** 193–201.
8. Park, J. H. and Y. S. Lee. 1996. Pathological study of an infectious lymphoid organ necrosis virus infection in penaeid shrimp, *Penaeus orientalis*. *Kor. J. Vet. Res.* **36:** 1013–1016.
9. Park, J. H., Y. S. Lee, S. Lee, and Y. Lee. 1998. An infectious viral disease of penaeid shrimp newly found in Korea. *Dis. Aquatic Organisms* **34:** 71–75.
10. Takahashi, Y., T. Itami, M. Maeda, N. Suzuki, J. Kasornchandra, K. Supamattaya, R. Khongpradit, S. Boonyaratpalin, M. Kondo, K. Kawai, R. Kusuda, I. Hirono, and T. Aoki. 1996. Polymerase chain reaction (PCR) amplification of bacilliform virus (RV-PJ) DNA in *Penaeus japonicus* Bate and systemic ectodermal and mesodermal baculovirus (SEMBV) DNA in *Penaeus monodon* Fabricus. *J. Fish Dis.* **19:** 339–403.
11. Tapay, L. M., E. C. Nadala, and P. C. Loh. 1999. A polymerase chain reaction protocol for the detection of various geographical isolates of white spot virus. *J. Virol. Methods* **82:** 39–43.
12. Van Hulten, M. C. W., M. Westenberg, S. D. Goodall, and Vlak. J M. 2000. Identification of two major virion protein genes of white spot syndrome virus of shrimp. *Virology* **266:** 227–236.
13. Wang, C. S., K. F. J. Tang, G. H. Kou, and S. N. Chen. 1997. Light and electron microscopic evidence of white spot disease in the giant tiger shrimp, *Penaeus monodon* (Fabricius), and the kuruma shrimp, *Penaeus japonicus* (Bate), cultured in Taiwan. *J. Fish Dis.* **20:** 323–331.
14. Wonfteerasupaya, C., J. E. Vickers, S. Sriurairatana, G. L. Nash, A. Akarajamorn, V. Boonsaeng, S. Panyim, A. Tassanakajon, B. Withyachumnarnkul, and T. W. Flegel. 1995. A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn *Penaeus monodon*. *Dis. Aquatic Organisms* **21:** 69–77.