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In situ Hybridization of a Megalocytivirus Using Nucleic Acid Probes against ATPase and the Major Capsid Protein of Rock Bream Iridovirus

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Systemic infections of maricultured fishes by *Megalocytivirus* species have occurred over a broad area in South Korea, causing extensive economic loss. We developed digoxigenin-labeled nucleic acid probes against the 230-bp ATPase and 311-bp major capsid protein (MCP) of rock bream *Oplegnathus fasciatus* iridovirus (RBIV) using polymerase chain reaction, and an *in situ* hybridization (ISH) method to detect *Megalocytivirus* in formalin-fixed tissues of mariculture species (rock bream, sea bass, and olive flounder). ISH-positive cells were abundant in the hematopoietic and connective tissues of various organs, while brain tissue showed little or no signal. The ISH procedure can become an important diagnostic tool in complement with histopathological methods, and advances epidemiological studies on the origin and distribution of *Megalocytivirus* in mariculture.

Key words: *Oplegnathus fasciatus*, *In situ* hybridization (ISH), Marine fish iridovirus, *Megalocytivirus*, Major capsid protein (MCP), ATPase

Introduction

Iridoviruses are large cytoplasmic DNA viruses with an icosahedral capsid approximately 200±50 nm in diameter. Their genome comprises a single linear dsDNA molecule. The family *Iridoviridae* is divided into five genera: *Iridovirus*, *Lymphocystivirus*, *Chloriridovirus*, *Ranavirus*, and *Megalocytivirus* (Chinchar et al., 2005). The lymphocystis disease virus (LCDV) is the most well-known member of the *Iridoviridae*; it affects fish, and causes lymphocystis in the olive flounder (*Paralichthys olivaceus*) and sea bass (*Lateolabrax japonicus*) (Wolf, 1988). However, LCDV does not cause fish death directly. *Megalocytivirus* was recently identified as a new genus in the family (Chinchar et al., 2005), and infectious spleen and kidney necrosis virus (ISKNV; He et al., 2002), which was isolated from the mandarin fish *Siniperca chuatsi* in China, is the type species of the genus. Viruses belonging to this genus have caused mass mortality in various marine and freshwater fish species, resulting in serious economic losses in Asian aquaculture (Inouye et al., 1992; He et al., 2000; Jung and Oh, 2000, Wang et al., 2003). The key characteristics of fish affected with these viruses are the systemic formation of enlarged cells and the necrosis of splenocytes and hematopoietic cells (Nakajima et al., 1998). Kawakami and Nakajima (2002) reported that a disease caused by red sea bream iridovirus (RSIV), a *Megalocytivirus*, occurred in 31 cultured fish species in Japan, including Perciformes, Pleuronectiformes, and Tetraodontiformes.

In Korea, *Megalocytivirus* causing high mortality in the rock bream also called Japanese parrot-fish (*Oplegnathus fasciatus*), in net cages was first

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detected in August and early September 1998 (Jung and Oh, 2000), and studies on PCR detection methods and asymptomatic infection have been carried out (Jeong et al., 2006). Recently, we sequenced the complete viral genome of RBIV isolated from the rock bream (Do et al., 2004), and reported that the *Megalocytivirus* isolates were subdivided into three subgroups based on the major capsid protein (MCP) gene, which is one of the most important genes for analyzing genetic relationships in the *Iridoviridae* (Do et al., 2005).

In situ hybridization (ISH) has emerged as a valuable technique for the easy and quick detection of virus-infected cells. Moreover, it is a sensitive method that detects the target nucleic acids using nonradioactive-labeled nucleic acid probes under light microscopy. Therefore, ISH has been applied to diagnosing diseases of aquatic organisms, including viruses from crustaceans (Mari et al., 1993, 1995; Durand et al., 1996; Walton et al., 1999), shellfish (Cochennec et al., 1996; Le Roux et al., 1999; Kleeman and Adlard, 2000), and fish (Biering and Bergh, 1996; Gregory, 2002).

In this study, we used an ISH method using nucleic acid probes against the ATPase and MCP of RBIV to examine tissues from various organs in rock bream, sea bass, and olive flounder infected with *Megalocytivirus*.

Materials and Methods

Species and tissue section preparation

From July to September of 2005, affected rock bream, sea bass, and olive flounder observed swimming abnormally in the Tongyeong area of South Korea were collected. All the visceral organs (liver, spleen, kidney, intestines, brain, heart, and gills) and muscle were fixed in neutralized buffered formalin (NBF), and pieces of these tissues were frozen in dry ice in the field or in a deep freezer in the laboratory.

For histological examination, the NBF-fixed tissues were dehydrated, impregnated, and embedded in paraffin wax using standard methods. Then, 4-5 μm sections were stained with hematoxylin and eosin (H&E) and periodic acid Schiff (PAS). Serial sections from the same tissue blocks were retained and placed on pretreated slides for ISH.

Virus extraction

For virus extraction, we used the High-Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) and AccuPrep Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea), as described in Do et al. (2005). The extracted viral DNA was used as the DNA template.

In situ hybridization of tissue sections

Serial sections for ISH were layered on poly-L-lysine-treated slides (Poly-prep™ slides; Sigma-Aldrich, St. Louis, MO, USA), placed standing on edge to dry at 55-60°C overnight, and stored at 4°C until use.

1) Probe labeling

Digoxigenin (DIG)-labeled probes were produced after extracting RBIV DNA from affected fishes. The specific primers developed were RBIV-A-P Forward (5'-GTAGTGATATCGGGCTCCGA-3') and RBIV-A-P Reverse (5'-CCGTTCTTGAACAGGTCCAT-3') for a region of ATPase and RBIV-M-P Forward (5'-C GTGATGGAGGGGATCTTAA-3') and RBIV-M-P Reverse (5'-GAAAAACGAGGCCGATCATA-3') for the major capsid protein (MCP). The expected products were 230 and 311 bp, respectively. The sequences of these DNA probes correspond to the sequence in the RBIV genome database (GenBank accession no. AY532614). For DIG-labeling and probe preparation, a mixed solution was prepared in one PCR tube containing 10×PCR buffer, 10 μL 15 mM $MgCl_2$, 2 μL of each forward primer (10 pmol/μL), 2 μL of each reverse primer (10 pmol/μL), 0.5 μL *Taq* polymerase (5 U/μL; Takara, Tokyo, Japan), 10 μL of PCR-DIG labeling mix or 10×dNTPs solution (for non-DIG-labeled probes), 2 μL of either DNA template or distilled water (DW, as a negative control), and DW to a volume of 100 μL. After an initial 5-min denaturation at 94°C, the PCR was run for 35 cycles of 30 sec at 94°C, 30 sec at 57°C, and 45 sec at 72°C, followed by a final elongation for 7 min at 72°C. In this study, the two primer sets were used in multiplex PCR (Fig. 1).

2) *In situ* hybridization (ISH)

The paraffin was removed from the sections by immersing them two or three times in xylene for 10 min, rinsing twice in 100% ethanol for 5 min, and then air-drying in a fume cabinet for 5 min. The sections were reacted by incubating the slides with 10 μg/mL proteinase K in 0.1 M Tris (pH 8.0) for 30 min at 37°C in a humid chamber. The proteinase K was drained off and the slides were rinsed for 3 min in Tris buffer (TB) in a Coplin jar. The slides were further dehydrated in 95% ethanol for 1 min followed by 1 min in 100% ethanol. The ethanol also inactivated the remainder of the proteinase K. The slides were air-dried for 5 min in a fume cabinet.

Hybridization buffer (50% formamide, 10% dex-

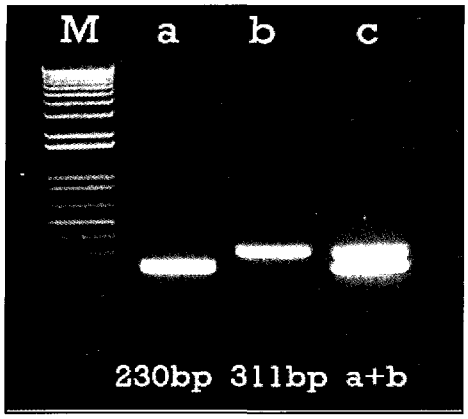

Fig. 1. PCR products, M, marker; a, ATPase region 230 bp; b, MCP region 311 bp; c, multiplex PCR product of a and b.

tran sulfate, 4×SSC, 250 μg/mL yeast tRNA, 1× Denhart's solution) stored at -20°C was allowed to warm to room temperature (RT) and mixed thoroughly. Hybridization mix was prepared by adding one volume of hybridization buffer to one volume of probe, giving a final concentration of at least 50 ng/μL of probe. The target virus DNA was denatured by incubating the slides for 5 min at 95°C and rapidly cooling them to the hybridization temperature. The slides were then hybridized overnight (18-20 h) at 42°C, and the excess probe was removed by washing the slide in a large volume of washing buffer (DIG Wash and Block Buffer Set; Roche), and then in washing buffer at 40°C for 10 min with gentle agitation to remove as much nonspecifically bound probe as possible. The tissue sections were then blocked in blocking buffer for 30 min at RT.

Specific probe binding in tissue sections was visualized using a sandwich consisting of sheep anti-DIG antibody, conjugated with alkaline phosphatase (AP; Anti-digoxigenin-AP; Roche). The antibody conjugate was diluted 1:50 in blocking buffer according to the manufacturer's recommendations, and incubated on slides for 1 h at RT in a dark, humid chamber. The antibody was drained off and the slides were washed in three changes of washing buffer over a period of 30 min. Detection buffer (DIG Wash and Block Buffer Set; Roche) was treated for 2 min before the substrate was added. BCIP/NBT substrate (Roche) was prepared as recommended by the manufacturer, and incubated on the slides for 1 h in a dark, humid chamber. A subsequent wash in 100 mM Tris-HCl buffer (pH 9.5) for 1 to 2 min was used to remove the excess substrate. The slides were mounted in buffered glycerol (pH 9.5) and observed. Bismarck brown Y stain was used occasionally.

Results

ISH in rock bream

The spleen and kidney showed the strongest reaction, followed by the intestine, liver, heart, and gill, while muscle displayed a weak reaction and the brain had little or no signal on the tissue slides. In the spleen, ISH-positive cells were distributed in the central region in several fish, but in most cases, these cells were observed throughout the spleen (Fig. 2B). In the kidney, ISH-positive cells were distributed throughout the section (Fig. 2C), and in the liver were located in or near the veins or sinuses (Fig. 2A). Intestine and stomach sections showed ISH-positive cells in the lamina propria, which consists of connective tissue and vessels. The heart contained many ISH-positive cells in the ventricle, and these cells were located at the inner surface of the ventricle and in the intercalary cardiac muscle bundle. Affected cells that could not be differentiated using PAS stain were identified on ISH (Fig. 3). Adipose tissue around the heart or intestine contained many ISH-positive cells. In the gills, ISH-positive cells were located in the central connective tissue of the gill filament, and some cells were observed at the tip of the gill lamella. Muscle showed a weak ISH-positive signal in the epimysium, and obvious signals were detected for the dermis and epidermis of the skin.

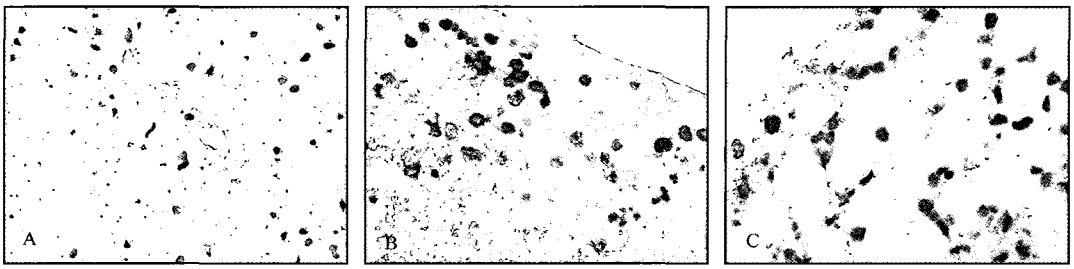

Fig. 2. Rock bream tissue after ISH. A, Liver (×200), Bismarck brown Y stained after ISH; B, Spleen (×400); C, Body kidney (×400).

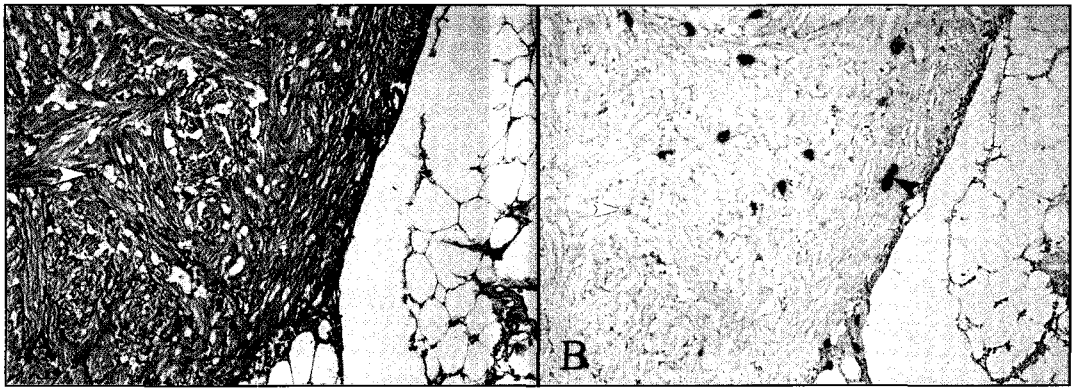

Fig. 3. Heart of rock bream. A, PAS stain (×200); B, Bismarck brown Y stained after ISH (×200). Arrowheads are same cells each black and white.

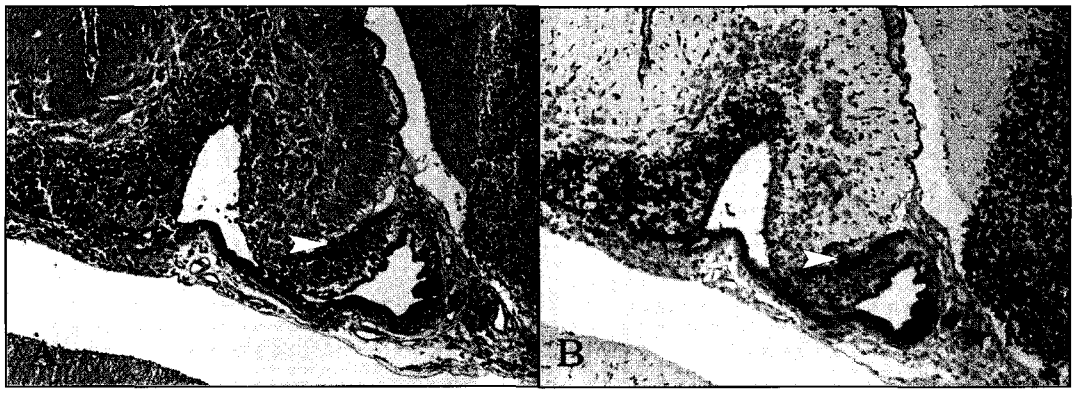

Fig. 4. Brain of rock bream. A, PAS stain (×200); B, Bismarck brown Y stained after ISH (×200). Arrowheads are same cells each black and white.

Most of the brain sections lacked an ISH-positive signal, although a few signal cells were stained in the outer layer of the cerebellum on rare occasions (Fig. 4).

ISH in sea bass

Compared to the rock bream, the spleen and kidney also showed strong signals after ISH, but the liver, heart, gill, intestine, and muscle contained fewer posi-tive cells and the sea bass cells were more spherical (Fig. 5A, 5B). The locations of the ISH-positive cells in various organs in sea bass were similar to those in rock bream, although the brain lacked an ISH-posi-tive signal.

ISH in olive flounder

As in the other two species, the spleen and kidney showed stronger signals than the other organs, and the number of ISH-positive cells equaled the number in rock bream, although the olive flounder cells were more spherical. Although the cell position was same as in the other two species, the ISH-positive signals in the liver, gill, heart, intestine, and muscle were weaker than in the rock bream (Fig. 6A, 6B), and no signal was detected in the brain sections.

Table 1 shows the ISH signal intensity in different organs of the three species. The negative control fish consistently gave negative results.

Discussion

The MCP and ATPase regions are highly conserved; in particular, the MCP gene is highly homologous in flounder iridovirus (FLIV), red sea bream iridovirus (RSIV), and sea bass iridovirus (SBIV), with 95 to 99% homology (Do et al., 2005). Therefore, we constructed nucleic acid probes against the MCP and ATPase for ISH. In this study, the nucleic acid probe proved successful in all three fish species, and the ISH signal tended to be similar in the same organs of each species. Therefore, our ISH method using nucleic acid probes is an effective diagnostic method for iridovirus disease in marine fishes.

The ISH method is an efficient tool for identifying

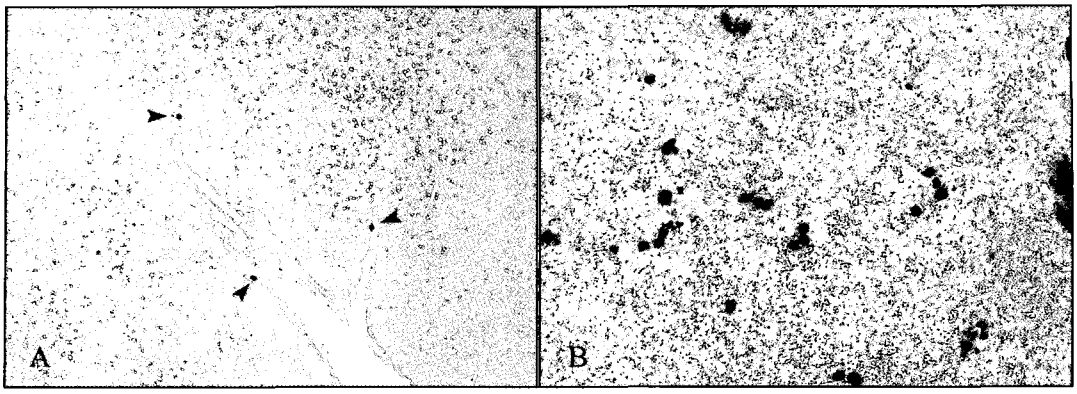

Fig. 5. Sea bass tissue after ISH. A, Liver (×200), arrowheads point ISH positive cells; B, Spleen (×400).

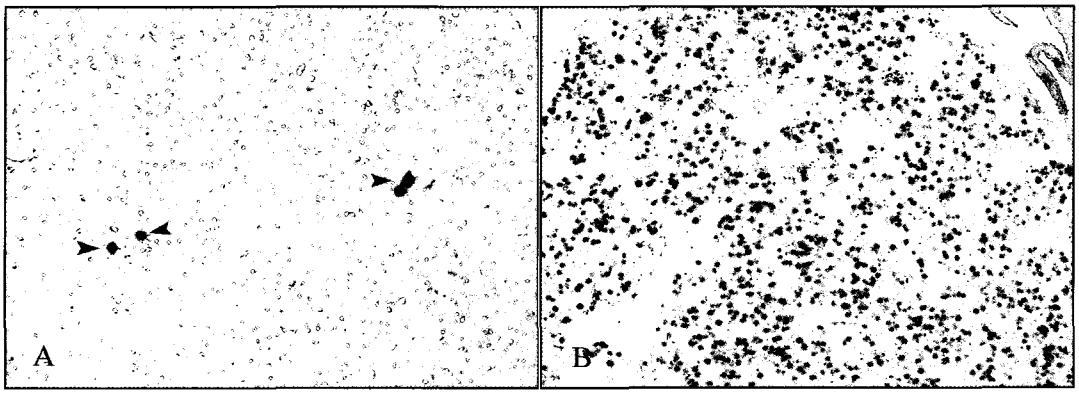

Fig. 6. Olive flounder tissue after ISH. A, Liver (×400), arrowheads point ISH positive cells; B, Spleen (×200).

Table 1. ISH signal intensity by organs in the rock bream, sea bass, and olive flounder

Organ / Fish	Gills	Brain	Liver	Spleen	Kidney	Intestines	Heart	Skin and muscle
Rock bream	++	+	++	+++	+++	++	++	+
Sea bass	+	–	+	+++	+++	+	+	+
Olive flounder	+	–	+	+++	+++	+	+	+

(+++, more than 20 cells; ++, between 5 and 20 cells; +, less than 5 cells; –, no signal at 200× magnification).

virus-infected cells. Small infected leucocytes that were no different from uninfected cells using standard histological methods could be observed as ISH-positive cells, as seen in Figures 3 and 4 comparing the PAS stain and ISH. In this study, ISH-positive cells were abundant in the hematopoietic and connective tissues in various organs, including the gills, intestines, eye, and skin. In the liver, ISH-positive cells were observed near or in sinuses or central veins, while in the heart ISH-positive cells were located at the inner surface of the ventricle or intercalary cardiac muscle bundle. In this study, only a few ISH-positive cells were observed in the rock bream brain, which is likely an effect of the blood-brain barrier (Patricia and Mercer, 1993). No ISH-positive cells were seen in brain tissue from sea bass or olive flounder.

Based on the tissue distribution of ISH-positive cells, the cells are presumed to be immigrant cells, such as plasma cells and monocytes (tissue macrophages), which are derived from myeloid and lymphoid stem cells, respectively, in higher vertebrates. Plasma cells immigrate into connective tissues and are abundant in lymphoid tissues along with macrophages. The stem cells that give rise to lymphoid and granular cells in fish are not known, although the thymus and kidney have been identified as lymphocyte-producing organs (Iwama, 1996). The spleen is a network of connective tissue in which leukocytes and red blood cells aggregate. The definite origin of the affected cells was not clarified in this study; splenic and hematopoietic dysfunction lead to the

production of abnormal blood cells, and anemia is manifested clinically as pale gills. The distribution of ISH-positive cells tended to be similar in all three fish species, although the ISH-positive cells in rock bream were larger than in sea bass or olive flounder, and the cells in sea bass and olive flounder were more spherical than in rock bream.

These results suggest that the morphological characteristics of the affected cells differ according to fish species, despite the similarity of the cells. In particular, bream have higher monocyte and granulocyte counts than other fish (Ikeda et al., 1986). Therefore, we postulate that the difference in pathogenicity among the species was attributable to the blood cell composition and physiological characteristics of the fish. Therefore, to examine the differences in mortality in fish species, it is necessary to examine host factors, such as blood cell composition and physiological characteristics.

Acknowledgements

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References

Biering, E. and Ø. Bergh. 1996. Experimental infection of Atlantic halibut, Hippoglossus L., yolk-sak larvae with infectious pancreatic nectosis virus: detection of virus by immunohistochemistry and *in situ* hybridization. J. Fish. Dis., 19, 405-413.

Chinchar, G., S. Essbauer, J.G. He, A. Hyatt and T. Miyazaki. 2005. Family iridoviridae. In: Fauquet, CM., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A., Eds. Virus taxonomy classification and nomenclature of viruses: eighth report of the international committee on the taxonomy of viruses. Academic Press, San Digo, 145-161.

Cochennec, N., F. Le Roux, F. Berthe and A. Gerard. 2000. Detection of *Bonamia ostreae* based on small subunit ribosomal probe. J. Invertebr. Pathol., 76, 26-32.

Do, J.W., C.H. Moon, H.J. Kim, M.S. Ko. 2004. Complete genomic DNA sequence of rock bream iridovirus. Virology, 325, 351-363.

Do, J.W., S.J. Cha, J.S. Kim, E.J. An, N.S. Lee, H.J. Choi, C.H. Lee, M.S. Park, J.W. Kim, Y.C. Kim and J.W. Park. 2005. Phylogenetic analysis of the major capsid protein gene of iridovirus isolates from cultured flounders *Paralichthys olivaceus* in Korea. Dis. Aquat. Org., 64, 193-200.

Durand, S., D.V. Lightner, L.M. Nunan, R.M. Redman, J. Mari and J.R.M. Bonaini. 1996. Application of gene probes as diagnostic tools for white spot baculovirus (WSBV). Dis. Aquat. Org., 27, 59-69.

Gregory, A. 2002. Detection of infectious salmon anaemia virus (ISAV) by in situ hybridization. Dis. Aquat.Org., 27, 59-66.

He, J.G., S.P. Wang, K. Zeng, Z.J. Huang and S.M. Chan. 2000. Systemic disease caused by an iridovirus-like agent in cultured mandainfish, *Siniperca chuatsi* (Basilewsky), in China, J. Fish Dis., 23, 219-222.

He, J.G., K. Zeng, S.P. Weng and S.-M. Chan. 2002. Experimental transmission, pathogenicity and physical-chemical properties of infectious spleen and kidney necrosis virus (ISKNV). Aquacuture, 204, 11-24.

Ikeda, I., H. Osaki and K. Sesaki. 1986. Blood Atlas of Fishes, Midorisyobo, Tokyo, 244-259.

Inouye, K., K. Yamano, Y. Maeno, K. Nakajima, M. Matsuoka, Y. Wada and M. Sorimachi. 1992. Iridovirus infectioun of cultured red sea bream, *Pagrus major*. Fish Pathol., 27, 19-27.

Iwama G. 1996. The Fish immune system. In: Organism, Pathogen, and Enviroment. Academic Press, San Diego, 43-45.

Jeong, J.B., L.J. Jun, K.Y. Park, K.H. Kim, J.K. Chung, J.L. Komisar and H.D. Jeong. 2006. Asymptomatic iridovirus infection in various marine fishes detected by a 2-step PCR method. Aquaculture, 255, 30-38.

Jung, S.J. and M.J. Oh. 2000. Iridovirus-like infection associated with high mortalities of striped beakperch, *Oplegnathus fasciatus* (Temminck et Schlegel), in southern coastal areas of the Korean peninsula. J. Fish Dis., 23, 223-226.

Kawakami, H. and K. Nakajima. 2002. Cultured fish species affected by red sea bream iridoviral disease from 1996 to 2000. Fish Pathol., 37, 45-47.

Kleeman, S.N. and R.D. Aclard. 2000. Molecular detection of *Marteillia sydneyi*, pathogen of Sydney rock oysters, Dis. Aquat. Org., 40, 137-146.

Le Roux F., C. Audemard, A. Bamaud and F. Berthe. 1999. DNA probes as potential tools for the detection of *Marteilia refringens*. Mar. Biotechnol., 1, 588-597.

Mari, J., J.R. Bonami and D.V. Lightner. 1993. Partial cloning of the genome of infectious hypodermal and haematopoietic necrosis, an unusual parvovirus pathogenic for penaeid shirimp: diagnosis of the disease using a specific probe. J. Gen.Virol., 74, 2637-2643.

Mari, J., D.V. Lightner, B.T. Poulos and J.R. Bonami. 1995. Partial clonig of the genome of an unusual shrimp parvovirus(HPV): use of gene probe in disease diagnosis. Dis. Aquat. Org., 22, 129-134.

Nakajima, K., K. Inouye and M. Sorimachi. 1998. Viral diseases in cultured marine fish in Japan, Fish Pathol., 33, 181-188.

Patricia, C.C. and K.L. Mercer. 1993. Cell and Tissue

Ultrastructure: A Functional Perspective. Freeman and Company, New York, 1-150.

Walton, A., H. Montanie, J.M. Arcier, V.J. Smith and J.R. Bonami. 1999. Construction of a gene probe for detection of P virus (reoviridae) in a marine decapod. J. Virol. Meth., 81, 183-192.

Wang, C.S., H.H. Shin, C.C. Ku and S.N. Chen. 2003. Studies on epizootic iridovirus infection among red sea bream, *Pagrus major* (Temminck & Schlegal), cultured in Taiwan. J. Fish Dis., 26, 127-133.

Wolf, K. 1988. Lymphocystis disease. In: Fish Viruses and Fish Viral Disease, K. Wolf, Ed., Cornell University Press, New York, 268-291.

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