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Presence of low level infected iridovirus in sea perch *Lateolabrax* sp. imported to Korea

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We have identified an iridovirus CH-1 from sea perch *Lateolabrax* sp. healthy externally and imported from China to Korea. In a comparison of the nucleotide sequences of the five different genomic regions, the CH-1 appears to be closely related to the ISKNV, IVS-1 and Ehime-1 strains detected in China, Korea and Japan respectively. In quantitative comparison of the viral DNA, level of CH-1 in tissue of imported fish was 10,000 times lower than that of IVS-1 strain presented in the infected rock bream *Oplegnathus fasciatus* of moribund stage. It allowed us to speculate the possibility of the asymtomatic iridovirus infection in the culturing sea perch. Such possibility of asymptomatic infection was supported by result of no appearance of dead fish with typical symptoms of iridoviral disease in keeping experiment of the imported sea pearch in laboratory for more than three weeks. Such asymptomatic infections with iridovirus were also found in spleen of the culturing and externally healthy sea perch of Korea by the presence of the iridoviral DNA in nested PCR.

Key words: Iridovirus, ISKNV, *Lateolabrax* sp., Asymptomatic infection, Nested PCR

Since the discovery of the first fish iridovirus, lymphocystis disease virus (LCDV), in 1962 (Walker, 1962), many new iridovirus-like pathogens have been reported from over 140 different species of fish worldwide (Essbauer and Ahne, 2001). The *Iridoviridae* family is subdivided into five genera, *Iridovirus*, *Chloriridovirus*, *Ranavirus*, *Lymphocystivirus* and *Megalocytivirus*, by the International Committee on Taxonomy of Viruses (ICTV) (Chinchar *et al*., 2005). Members of *Megalocytivirus* produce characteristic basophilic inclusions in hypertrophied cells in many organs and have been responsible for serious problems in modern aquaculture, fish farming, and wildlife conservation because of its epidemic morbidity and ability to cause mortality (Gibson-Kueh *et al*., 2003). The same clinical signs and pathology occur in infections due to red sea bream iridovirus (RSIV) (Nakajima and Sorimachi, 1994), infectious spleen and kidney necrosis virus (ISKNV) (He *et al*., 2000), rock bream iridovirus (RBIV) (Do *et al*., 2004), grouper sleepy disease virus (GSIV) (Chua *et al*., 1994) and iridovirus IVS-1 (Jeong *et al*., 2003), all of which affect species farmed for food. Additionally, African lampeye iridovirus (ALIV) and dwarf gourami iridovirus (DGIV) (Sudthongkong *et al*., 2002) also have been reported to affect farmed ornamental fish.

Iridoviruses are notable for their variability in infecting of vertebrate and invertebrate hosts (Williams, 1996). Understanding the degree of variations in the pathogen would be critical for under-

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standing the biology, evolution, and control of iridoviral diseases (Goldberg *et al*., 2003). As an one of the variations, Hanson *et al*. (2001) have reported the long-term largemouth bass virus (LMBV) persistence using a primary PCR in the largemouth bass (*Micropterus salmoides*) population after the mass mortality of largemouth bass. It is possible that LMBV is maintained as a persistent infection, as has been suggested for infectious hematopoietic necrosis virus (IHNV) (Amend, 1975; Drolet *et al*., 1995; Kim *et al*., 1999).

Recently, we obtained iridovirus CH-1 detected from the imported sea perch (*Lateolabrax* sp.) from China and characterized the nucleotide sequences of a specific region, named K2, located between the DNA polymerase (DPOL) and the ribonucleotide reductase small subunit (RNRS) gene. Interestingly, these sampled fish were not showed clinical signs of disease externally (Jeong *et al*., 2006). In here, we describe the iridoviruses infected subclinically or asymptomatically in fish imported from foreign country and the DNA sequences of some genomic regions for the molecular comparison with iridoviruses isolated in other laboratories. The results of this study are suggesting the potential risk of losses of culturing fishes associated with iridovirus in the world including China and Korea.

Materials and Methods

Virus

In previous studies, we have reported (Jeong *et al*., 2003) the representative iridovirus of Korea, iridovirus IVS-1, in rock bream (*Oplegnathus fasciatus*) of 100 g body weight respectively suffering from the typical iridovirus infection in september of 2000 from the aquatic farms of the South Sea in Korea. We also have obtained iridovirus CH-1 from the imported sea perch of 5-7 g body weight as a live fly from China by screening the presence of RSIV using PCR with the primers derived from the MCP gene (Table 1). These fish were imported as a live fry in the spring of 2000 from China and healthy externally (Jeong *et al*., 2004).

Sample processing

Ten sea perch were imported from China were killed and tested the presence of iridovirus in spleen

Table 1. Genomic regions used for the diagnosis of iridovirus infection with PCR method

Genomic region	Primer	Oligonucleotide sequence (5' to 3' direction)	Expected size of amplicon	Reference
RNRS gene	VF VR	GCATGTATGCTGTTTAGACA GAGCATCAAGCAGGCGATCT	187 bp	Oshima et al. (1998)
Pst I fragment	2F 2R	TACAACATGCTCCGCCAAGA GCGTTAAAGTAGTGAGGGCA	563 bp	Kurita et al. (1998)
ATPase gene	3F 3R	CAAACCACAGCGCGGCAAGT AGTAGCGCACCATGTCCTCC	563 bp	Kurita et al. (1998)
DPOL gene	4F 4R	CGGGGGCAATGACGACTACA CCGCCTGTGCCTTTTCTGGA	567 bp	Kurita et al. (1998)
Flanking region of MCP gene	M1F M1R	GAGAGACCCCAACACGAC ACCTGGTGGCTCCAGTGC	1828 bp	In this study
MCP gene	M2F M2R	ATAACGACCAGTTCAAAC GGCGGCGACAATGCCGTG	908 bp	In this study

by PCR with M1F/M1R primer set (Table 1) and another ten sea perch were kept in 500 ℓ tank of laboratory at 25℃ for 3 weeks as a keeping experiment to determine if there is further progressing of disease. After 3 weeks of keeping time, healthy fish without external signs of iridovirus infection, were collected the spleens and stored at -80℃ until used. After thawing, spleens of the same sample were pooled and homogenized in PBS (0.1 M, pH 7.3) at a 1:10 dilution (w/v) and were then centrifuged at 300 x g for 5 min, followed by centrifugation at 2600 x g for 10 min. After confirmation of iridovirus infection by PCR in the collected supernatants, they were used as the tissue homogenates in the experiments of this study.

Challenge experiments

Sea perch (200 g b.w.) culturing in aquatic farms were sampled and analyzed the infection of iridovirus by nested PCR. After 3 weeks of acclimation at 25℃ in laboratory, 10 sea perch were challenged by an intramuscular injection of 0.1 ㎖ of a 0.45-㎛ (pore size) filtered spleen homogenate infected by CH-1 (100 ㎍ spleen homogenate / ㎏ b.w.) and another 10 sea perch were also challenged by iridovirus IVS-1 (0.1 ㎍ spleen homogenate / ㎏ b.w.) (Jeong *et al*., 2003) as positive controls. After challenge, all fish were maintained at 25℃ in 500 ℓ tanks for 3 weeks. Water was changed daily and fed with commercially prepared pellets once a day. Mortalities and all survivors at the termination of experiments were tested by PCR as described below.

Isolation of viral nucleic acids

For DNA isolation, samples of about 20 ㎎ of spleen from diseased fish were homogenized in 355 ㎕ of TE buffer (100 mM Tris-HCl, 10 mM EDTA) using motor for pellet pestle (Sigma-Aldrich, Co., Ltd.) and centrifuged at 8000 g for 10 min. Supernatants were treated with 40 ㎕ of 10% SDS and 5 ㎕ of 20 ㎎/㎖ proteinase K (Boehringer Mannheim, Germany) for 1 hr at 37℃. After three extractions with phenol-chloroform, the DNA was precipitated with ethanol in the presence of 0.3 M sodium acetate, redissolved in 50 ㎕ TE buffer, and stored at -80℃ until use.

PCR

Five oligonucleotide primer sets were synthesized (Bioneer Co., Taejon, Korea) based on the nucleotide sequence of the RNRS gene, the Pst Ⅰ fragment, the ATPase gene, the DPOL gene and the MCP gene respectively (Table 1). PCR amplification was carried out in a 50 ㎕ reaction mixture containing the extracted viral nucleic acids (100 ng of the extracted total nucleic acids), 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001 % w/v gelatin, 0.5 % Tween-20, 200 ㎛M of each dNTP, 1 ㎛M of each primer, 1.25 U AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CT, USA) in a Perkin-Elmer 2400 thermal cycler (Perkin-Elmer). After 2 min of predenaturation at 94℃, the mixtures were incubated for 30 cycles at 94℃ for 30 s, 55℃ for 30 s and 72℃ for 30 s, followed by an extension period at 72℃ for 7 min. For nested PCR, we used the same PCR condition with primary PCR but 0.5 ㎕ of the reacted mixture taken from the tube of primary PCR as a template and internal primer pairs corresponding to the amplicons of the primary PCR. To determine the detection limit of PCR, the extracted viral nucleic acids diluted 10 fold serially were used as template. Dilution rate of each different template that can produce the amplicons visible in agarose gel electrophoresis after PCR was compared.

DNA sequencing

The PCR products were purified by agarose gel

electrophoresis using a Prep-A-Gene DNA Purification systems (Bio-Rad Laboratories, Hercules, CA, USA) and cloned into the TOPO-TA vector following the instructions of the manufacturer (Invitrogen Co., Carlsbad, CA, USA). The cloned fragment was sequenced using the Big Dye Terminator Cycle DNA Sequencing Kit (ABI PRISM, PE Applied Biosystems, Foster City, CA, USA) and an automatic sequencer. Nucleotide sequences and the deduced amino acid sequences were compared based upon a gene alignment using the BioEdit program (Version 5.0.9. Department of Microbiology, North Carolina State University, Raleigh, NC, USA).

Survey for asymptomatic infection

In spring of 2003, sea perch of ten market size groups (0.5-1 ㎏ b.w.) and five small size groups (10-20 g b.w.) were collected and tested for iridovirus infection by nested PCR respectively. Oligonucleotide primer sets for primary (M1F/M1R) and nested (M2F/M2R) PCRs were synthesized (Bioneer Co., Taejon, Korea) based on the nucleotide sequences of the MCP gene region (Table 1). For keeping experiment, fishes were kept in 500 ℓ tank of laboratory at 25℃ for 3 weeks as stated above.

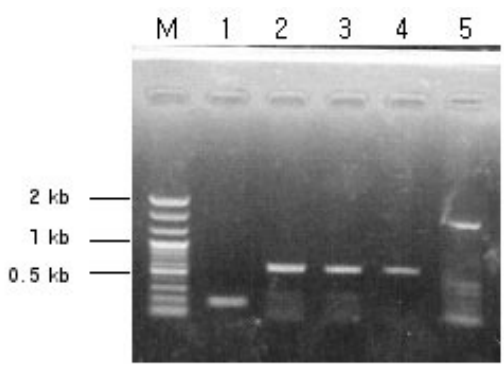

Fig. 1. Detection of the DNA isolated from iridovirus CH-1 by PCR with different RSIV specific primers. Product of PCR amplification, lane 1, 2, 3, 4, 5, with primer sets VF/VR, 2F/2R, 3F/3R, 4F/4R, M1F/M1R respectively. M, 100 bp DNA ladder.

Results

Detection of iridovirus in sea pearch imported from China

For PCR, the primers that have been reported to detect the specific genes of RSIV in other laboratories (Kurita *et al*., 1998) and designed in this study were used (Table 1). PCR with these five sets of primers for the RNRS gene, the *Pst* Ⅰ fragment, the ATPase gene, the DPOL gene and the MCP gene were specific the iridovirus IVS-1 used as a positive control and produced fragments of the expected sizes, 187, 563, 563, 567 and 1,828 bp respectively (data not shown). As the same way, we found that the resulting amplicons with five different primer sets, matched exactly the expected sizes from the viral DNA templates obtained from the imported sea pearch (Fig. 1). In five sea pearch imported from China in 2000, except one sample, all four samples appeared to be infected by iridovirus.

Quantification and pathogenicity of iridovirus CH-1 in fish

In PCR, it appears that the density of the bands produced by the amplicon using CH-1 was always lower than that of the bands produced by IVS-1 of positive control. The density of the band produced by the amplicon using CH-1 was 10,000 times less than that of IVS-1 as shown by serial-dilution PCR with 4F/4R primers (Fig. 2A). The same PCR result was obtained with primer pairs, 2F/2R, which was designed by Kurita *et al* (1998) (Fig. 2B). The concentration of genomic DNA of the CH-1 in the individual fish analyzed appeared to be similar one another by this serial-dilution PCR. In terms of pathogenicity in host, we could not observe any

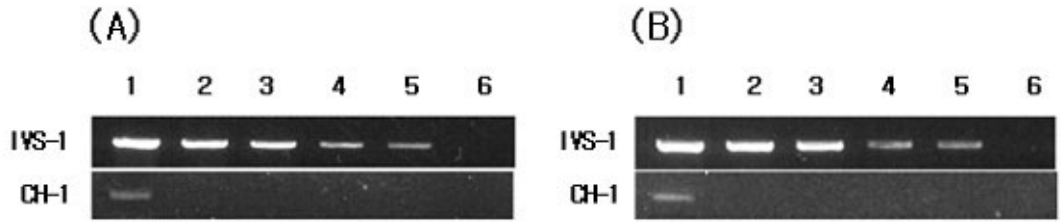

Fig. 2. Detection limit of genomic DNA of RSIVs by PCR. The PCR was performed with the primer pair on a serially diluted (1:10) viral DNA template. (A) product of PCR amplification with primers 4F/4R; (B) product of PCR amplification with primers 2F/2R; Lane 1-6, with serially-diluted template DNA prepared from the pool of three infected spleens from 10^{-1} to 10^{-6} fold for CH-1 and IVS-1 strain, respectively.

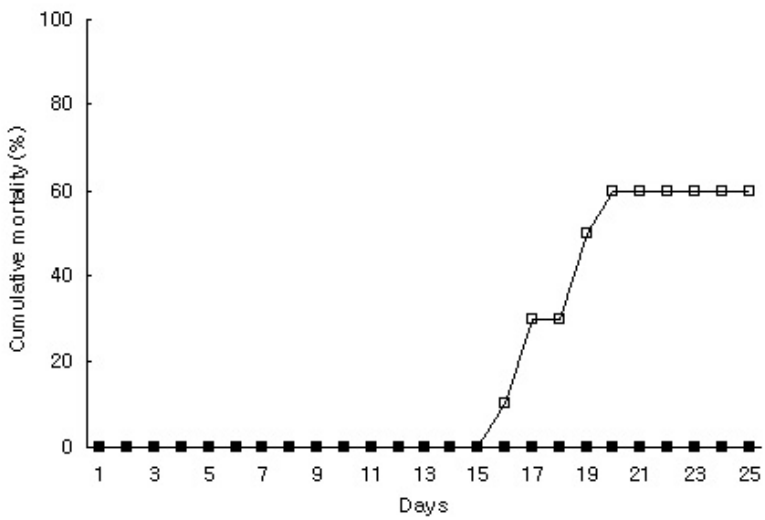

Fig. 3. Cumulative mortality (%) of sea perch after challenge with the iridovirus IVS-1 (□) and CH-1 (■). Sea perch (200 g b.w.) culturing in aquatic farms were challenged by intramuscular injection of 0.1 ㎖ of tissue homogenate corresponding to spleen 0.1 ㎍ infected by iridovirus IVS-1 or spleen 10 ㎍ infected by iridovirus CH-1.

dead fish in the groups challenged with iridovirus CH-1 (100 ㎍ spleen homogenate / kg b.w.) compared to the positive control groups showing 60% cumulative mortality within 20 days of challenge with iridovirus IVS-1 type (0.1 ㎍ spleen homogenate / kg b.w.) (Fig. 3).

Comparison of the nucleotide sequences

PCR fragments obtained from five different regions of the iridovirus CH-1 were sequenced and compared with those of the corresponding regions of other reported iridovirus strains. Although the differences in nucleotide sequences rarely resulted in the change of amino acid, the CH-1 showed differences in nucleotide sequences compared with those of the reference sequence of RSIV Ehime-1, iridovirus IVS-1 or ISKNV (Table 2). In comparing of the nucleotide sequences, CH-1 showed the highest identity of the DPOL region, 100%, with that of IVS-1 but showed the lowest identity of the *Pst* Ⅰ

fragment, 91.4%, with that of IVS-1. In the substitution of nucleotide sequences for amino acids, the DPOL gene was presented fewer changes than those of other four genes (Table 2).

Asymptomatic infection of iridovirus in sea pearch of Korea

In Mar of 2001, sea perch samples of market size (0.5～1 ㎏, b.w.) and small size (10～20 g, b.w.) obtained from each different batch were tested for iridovirus infection after collection. We could not find any positive fish by primary PCR with any of the primers used in this study, but three of ten sample groups of market size were positive by nested PCR, giving amplicons of the MCP of the expected length (Fig. 4). Such iridovirus infection determined by nested PCR was also found in two of five sample groups of small size cultured in aquatic farms (data not shown). Nucleotide sequence of the cloned amplicon of the MCP region in nested PCR showed 100% identity with that of the IVS-1.

Discussion

We found iridovirus CH-1 type in PCR with primer sets against the RNRS gene, *Pst* Ⅰ fragment, ATPase gene, DPOL gene and MCP gene (Table 1) from the spleens of sea perch (*Lateolabrax* sp.) imported from China as a live fry for culturing in the aquatic farms of Korea in 2000. Interestingly, in PCR, it appears that the density of the bands produced by the amplicon using CH-1 infected spleen was always lower than that of iridovirus IVS-1. In the detection limit analysis of PCR with 4F/4R primer set, the concentration of genomic DNA of CH-1 in the spleens of infected sea perch was 10,000 times less than that of IVS-1 (Fig. 2). The same PCR result was obtained with primer set, 2F/2R, which was designed by Kurita *et al*., (1998)

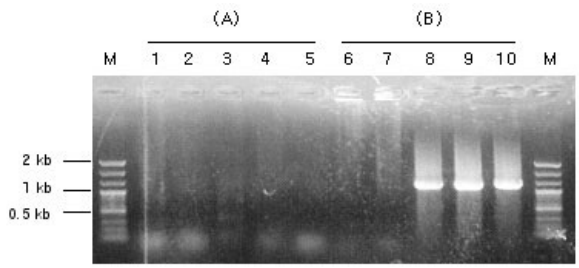

Fig. 4. Detection of iridoviruses in five sea perch (*Lateolabrax* sp.) sampled randomly from markets of Korea by primary PCR with M1F/M1R (A) and by nested PCR with M1F/M1R and M2F/M2R (B).

Table 2. Percentage similarities of the DNA nucleotides (amino acids) of the various genomic regions of iridovirus CH-1 compared with those of ISKNV, RSIV Ehime-1 or IVS-1

Iridovirus isolates	Fish	Reference	Homology with iridovirus CH-1 isolate (%)				
			RNRS gene	*Pst* I fragment	ATPase gene	DPOL gene	MCP gene
ISKNV	Mandarin	He et al.	97.3	94.1	96.4	95.3	94.1
	fish	(2001)	*(93.8)		(98.8)	(97.1)	(98.2)
RSIV	Red sea	BD143114	93.8	93.1	96.0	98.3	94.3
Ehime-1	bream	(GenBank no.)	(96.2)		(100)	(98.9)	(97.8)
IVS-1	Rock	Jeong et al.	91.8	91.4	96.2	100	94.5
	bream	(2003)	(91.7)		(98.3)	(100)	(98.2)

* ( ) : Percentage similarities of amino acids.

and used for detection of iridovirus in this study. Similar low levels of viral DNA have been reported recently in aquatic animals infected by nodavirus, the infectious hematopoietic necrosis virus (IHNV), and white spot syndrome virus (WSSV) (Isshiki *et al*., 2001; St-Hilaire *et al*., 2001; Pongmaneerat *et al*., 2001; Withyachumnarnkul *et al*., 2003). For iridoviruses, there have been some studies that analyzed the presence of viral DNA in different organs during an infection with RSIV (Sano *et al*., 2002), or less than 100,000 times of viral DNA in the tissues of asymptomatically infected rock bream compared with that of moribund fish infected clinically (Jeong *et al*., 2006).

With quantitative comparison of the viral DNA in infected tissues, it would be necessary to note that we could not observe the typical symptoms of RSIVD or mass mortality in the sea perch that we sampled and had kept for three weeks in the laboratory at 25℃ for analysis to observe the progressing of diseases by iridovirus CH-1. Additionally, as shown in Fig 3, no mortality was found in challenge experiment even with 1000 times higher concentration of CH-1 than that of IVS-1 detected from the rock bream infected clinically and used to challenge for the positive control group. Thus, even though iridovirus CH-1 was made in April rather than in late summer or early autumn known season for outbreaks of RSIVD, still it would be worthwhile to consider these results as evidences showing the presence of an asymptomatic iridovirus infection in sea perch of China that might be demonstrated by a very weak band in primary PCR or positive only in nested PCR. Such asymptomatic infections of iridovirus, as shown in Fig 4, were also found in externally healthy sea perch of fingerlings or market size by nested PCR and agreed to the previous results (Jeong *et al*., 2006). Thus, asymptomatic infection of iridoviruses in fish is not restricted to China but should be considered to be potential problems of aquatic industries or wildlife around the world.

In our preliminary experiments for the induction of asymptomatic infection into the clinical infection, we have used the injection of immune suppressive agents, lowered dissolved oxygen, and fluctuated water temperatures against the sea perch infected iridovirus asymptomatically. Unfortunately, any evidences considered as a potential trigger for the explosion of asymptomatic infection to the clinical infection were not found (data not shown). High-density rearing reported to induce white sturgeon iridovirus disease among asymptomatic carriers of white sturgeon (*Acipenser transmontanus*) have not been applied (Drennan *et al*., 2005). Certainly more specific other studies would be needed to determine whether the mechanism of infection of iridovirus CH-1 is different from that of other RSIVs, or whether persistent infection without mass mortality in fish, only during the winter season, is possible.

In a comparison of the nucleotide and amino acid sequences, CH-1 showed very high level of identity with other three iridovirus strains of *Megalocytivirus* genus, ISKNV, Ehime-1 and IVS-1 isolated in Asian countries (Table 2). Additionally, no differences were found in the nucleotide sequence comparison of the MCP region between IVS-1 induced mass mortality and another iridovirus detected by nested PCR from culturing sea perch in Korea. Thus, at least in the genomic regions analyzed in this study, we could not find any specific genomic changes that can discriminate the asymptomatically infected one from the symptomatically infected iridoviruses.

In summary, the results showed that iridovirus was detected in sea perch externally healthy whether these fish were imported from China or culturing in Korea. Further comparative studies

between different iridoviruses showing symptomatic and asymptomatic infection might be needed to obtain a more accurate molecular biological characterization of iridovirus and an understanding of the pathological mechanisms of the various iridoviruses.

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