Enormous Journal of Medical Sciences and Current Research

Research Article

Immunological Prevention of an Emerging Red Sea Bream Iridovirus [RSIV] in Cage-Cultured Spotted Mandarin *Siniperca Scherzeri* in Dandong, Northeast China

Wenfeng Zhang[1,2], Youyong Dong[1,2], Shaoping Weng[1,2], Chuanfu Dong[1,2*]

[1]State Key Laboratory for Bio-control, School of Life Sciences Sun Yat-sen University, China
[2]Institute of Aquatic Economic Animals and Guangdong Province Key Laboratory for Aquatic Economic Animals, Sun Yat-sen University, China

***Corresponding author:** Dr. Chuanfu Dong, School of Life Sciences; Sun Yat-sen University No. 135, China

Received: October 3, 2021

Accepted: October 15, 2021

Published: October 17, 2021

Abstract

Red sea bream iridovirus [RSIV] is one of the most important causative agents in marine finfish industry, and RSIV was documented rarely in freshwater fish. Here, a novel RSIV, designated as Megalocyti-KD1201, was isolated and characterized from a natural mass mortality of cage-cultured spotted mandarin *S. Scherzeri*, one of the seven fish species in genus *Siniperca*, in Dandong, Northeast China. By phylogenetic analysis, Megalocyti-KD1201 was classified into a distinct RSIV clade, in which clade an early well-known genome-sequenced RSIV-Ehime-1 isolate in Japan was included. Vaccination trials showed that inactivated whole cell vaccines [Megalocyti-KD1201 strain] conferred effective protection to vaccinated spotted mandarin, and greater than 90% protection were obtained in two field tests in cage-cultured spotted mandarin during 2016-2018. By contrast, less than 15% survivals were obtained in non-vaccination group. Vaccine inoculation showed a highly efficient treatment to prevent natural outbreak of RSIV diseases in cultured spotted mandarin.

Key words: Spotted mandarin *Siniperca Scherzeri*; Megalocytivirus; Red sea bream iridovirus [RSIV]; inactivated vaccine; Field test; Immunoprotection assessment.

Introduction

Megalocytivirus, a newly defined piscine iridovirus in family Iridoviridae, is one of the most alarming causative agents in aquaculture industry worldwide [Kurita and Nakajima, 2012; Subramanian et al., 2012]. The viruses could infect a wide range of marine and freshwater bony fish, well-known as red sea bream *Pagrus major,* rock bream *Oplegnathus fasciatus,* numerous kinds of groupers *Epinephelus spp.*, turbot *Scophthalmus maximus L* and mandarin *Siniperca chuatsi* [Kurita and Nakajima, 2012; Subramanian et al., 2012]. According to nucleotide variation of the major capsid protein [MCP] gene, the most conserved core gene in iridovirus [Eaton et al., 2007; Sudthongkong et al., 2002], megalocytiviruses can be mainly classified into three clades, which are represented by red sea bream iridovirus [RSIV], turbot reddish body iridovirus [TRBIV] and mandarin infectious spleen and kidney necrosis virus [ISKNV], respectively [Dong et al., 2010; Imajoh et al., 2007; Shuang et al., 2013]. The RSIV includes the megalocytiviruses that infect most marine fish from the order Perciformes. The TRBIV particularly infects flatfish species such as turbot *Scophthalmus maximus* and flounder *Paralichthys spp.* The ISKNV includes almost all freshwater megalocytiviruses [Dong et al., 2010; Go et al., 2006; Shuang et al., 2013; Tanaka et al., 2014]. In our previous report, RSIV-like virus showed highly virulent to freshwater mandarin S. chuatsi under artificial infection [Dong et al., 2010]. RSIV-associated outbreaks were also recorded in cage-cultured mandarin in South China [Dong et al., 2013b]. However, natural outbreak of RSIV has been reported rarely in other freshwater fish species.

Spotted mandarin *S. scherzeri,* also known as golden mandarin in South Korea [Shin et al., 2014], is one of the seven species in genus *Siniperca*. Spotted mandarin is a precious freshwater food fish species in Yalu River, an international river between China and North Korea. The wild spotted mandarin juveniles are captured from Yalu River for artificial cage-culture and a relative large-scale cage-culture spotted mandarin industry is formed in Dandong, a famous border city location near the outlet of Yalu River to Yellow Sea in Northeast China. According to the local fish farmers that mass mortalities of spotted mandarin occurred throughout cage-cultured spotted mandarin industry in Dandong when culture water temperature was over 25°C, however, the causative agent remained unclear for a long time as early since 2009 [data not shown]. In this study, we confirmed the causative agent as an unusual RSIV by molecular detection, spleen tissue-based histopathology and immunohistochemistry, cell culture-based virus isolation and indirect immunofluorescence assay, and transmission electron microscope observation. Moreover, vaccination trials of an inactivated whole cell vaccine were assessed to prevent this viral disease.

Citation: Wenfeng Z, Youyong D, Shaoping W, Chuanfu D. Isolation and Immunological prevention of an emerging red sea bream iridovirus [RSIV] in cage-cultured spotted mandarin *Siniperca Scherzeri* in Dandong, Northeast China. **ejmscr 2021; 1(5):1025**

Materials and methods

Cell line and antibody

Mandarin fish fry [MFF-1] cell line was developed and characterized in our laboratory, and routinely cultured in Dulbecco's modified Eagle's medium [DMEM] containing 10% fetal bovine serum [Invitrogen, USA] at 25 °C [Dong et al., 2008]. Mouse anti-ISKNV monoclonal antibody of 2D8 was prepared, characterized and kept in our laboratory [unpublished data by Dong et al.]

Fish samples and virus identification

Diseased spotted mandarin with body weight of 30-120 g/fish was collected in ice-frozen and taken back by air transport from a local spotted mandarin farm in Dandong to our laboratory in Guangzhou for pathogen identification. For virus detection and isolation, spleen and kidney tissues were collected and homogenized with 9 volumes of sterile phosphate buffered saline [PBS, pH 7.4]. After centrifugation at 10,000 g at 4 °C for 10 min, the supernatant was collected and filtered using 0.22 μm filter membrane [Millipore, USA]. One hundred microliters of filtered suspension were used for tissue DNA extraction according to Tissue DNA Extraction Kit [Omega, China]. The prepared tissue DNA was used as template for PCR-based virus detection using ISKNV/RSIV-specific universal primer set [MCP-F: 5'-TCATTGTCATCATCATGTCTGC-3'; MCP-R: 5'-AGACACACGGGGCAATC-3'] as our previous report [Dong et al., 2010]. The PCR products were selected to be cloned into pGEM-T Easy vector for sequence determination on a 377 DNA sequencer. The assembled sequence was subjected to run BLAST in the NCBI nr database. MCP sequences of some typical megalocytiviral isolates available in GenBank/EMBL nr database were selected for phylogenetic tree construction by neighbor-joining analysis with the MEGA5.0 program [Tamura et al., 2007]. Bootstrap sampling was resampling 1000 replicates.

Histopathology and immunohistochemistry

Spleen tissue from moribund spotted mandarin [Fig. 1A] was collected and fixed with 4% paraformaldehyde. The fixed tissue samples were dehydrated in a gradient of ethanol–xylenes, embedded in paraffin wax and sectioned to a 4-μm thickness. The sections of tissue samples were deparaffinized in xylene, rehydrated through a gradient of ethanol solutions. Some sections were stained with hematoxylin and eosin [H&E] for histopathology observation and some were used for immunohistochemistry [IHC] examination. For immunohistochemistry analysis, sections were incubated with mouse anti-ISKNV monoclonal antibody [2D8], followed by Horseradish-peroxidase [HRP]-conjugated goat anti-mouse IgG [Invitrogen] and then developed with DAB [Diaminobenzidine] staining kit for microscopic examination.

Virus isolation in MFF-1 cells

Confluent MFF-1 cells in a 25-cm2 flask were added 100 μl filtered tissue homogenate supernatant and incubated at 25 °C. The inoculated MFF-1 cells were observed daily until appearance of typical cytopathic effect [CPE] as previously descripted [Dong et al., 2010]. The infected MFF-1 cells with typical CPEs were harvested into -80 °C at least for 24 h and then thawed at room temperature for another round infection. After thrice viral passages in MFF-1 cells, the viral titer was determined using the 50% tissue culture infective dose [TCID50] method in 96-well culture plates [Reed and Muench, 1938]. The isolated virus in MFF-1 cells was designated as Megalocyti-KD1201. Immunofluorescence assay [IFA] was performed to characterize the infected MFF-1 cells in a 48-well tissue plate [Dong et al., 2008], using mouse anti-ISKNV monoclonal antibody 2D8 as the first antibody and Alexa fluor488 conjugated-donkey anti-mouse IgG as secondary antibody [Life Science, USA]. The stained MFF-1 cells were visualized under a Nikon inverted fluorescence microscope [Nikon, Japan].

Fish samples and virus identification

Confluent MFF-1 cells in a 25-cm2 flask were infected with Megalocyti-KD-1201 at an MOI of 1.0. After advanced CPEs appearance, the infected cells were collected by centrifugation at 2,000 g at room temperature for 3 min. The supernatant was removed and the harvested cells were fixed with 2.5% glutaraldehyde in 0.1 M PBS, and then re-fixed containing 2.0% osmium tetroxide in 0.1 M PBS. Ultrathin sections were stained with uranyl acetate-lead citrate and examined on a Philips CM10 electron microscopy as a previous report [Dong et al., 2010].

Vaccine preparation and vaccination tests

Megalocyti-KD1201 was propagated in MFF-1 cells and harvested into -80°C at least for 24 h. The virus suspension was thawed at room temperature and the viral titer was determined as abovementioned $TCID_{50}$ mothed. The virus was added with formalin to a final concentration of 0.1% at 4°C for 8 days. The inactivated virus was diluted to the final viral concentration of 106.5$TCID_{50}$ / 0.1 ml and then emulsified as water in oil as our previous description [Dong et al., 2013a]. The prepared vaccine was kept at 4 °C until use. Before vaccination, a meal feeding was skipped. In the same fish farm, numerous spotted mandarin were performed vaccination by intramuscular injection with 0.1 ml / fish and others were set as non-vaccination control. Vaccination and non-vaccination fish were cultured in adjacent cages and implemented the same daily aquaculture administration. During the field tests, moribund fish were sampled for RSIV detection by conventional PCR [Dong et al., 2010].

Results and discussion

Isolation of RSIV in cage-cultured spotted mandarin

Two batches of fish samples from natural massive mortalities of spotted mandarin were detected by PCR amplification using a megalocytivirus-specific universal primer set [Dong et al., 2010] and all samples showed positive amplification [data not shown]. Histopathology showed that numerous normal enlargement cells were observed in infected spleen tissue [Fig. 1B]. Using a megalocytivirus specific monoclonal antibody of 2D8, immunohistochemistry also showed that numerous brown-stained hypertrophic cells were developed in infected spleen tissue [Fig. 1C]. Both histopathology and IHC suggested that the spotted mandarin died of megalocytivirus infection. The filtered tissue supernatant was inoculated in MFF-1 cells. Typical CPEs, characterization with numerous rounding cells, were observed at 90 hours post infection [Fig. 2A]. IFA showed that strong green fluorescent signals were observed infected MFF-1 cells [Fig. 2B]. TEM also showed numerous spherical viral particles distributing in the cytoplasm of infected MFF-1 cell [Fig. 3]. All these data showed that the sampled spotted mandarin was infected with megalocytivirus, and a new megalocytivirus was isolated through cell culture-based viral isolation and designated as Megalocyti-KD1201. Furthermore, Basic Local Alignment Search Tool [BLAST] using

NCBI nr databases showed that Megalocyti-KD1201 is an RSIV but not ISKNV or TRBIV-close megalocytivirus.

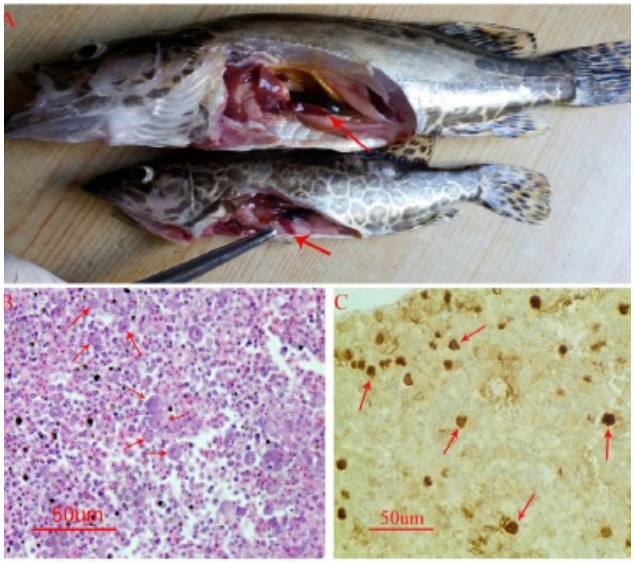

Figure 1: **Histopathology and immunohistochemistry analysis of the spleen tissue of moribund spotted mandarin.**
A) Autopsy of natural outbreak of spotted mandarin with swollen spleens [arrow indicate].
B) Histopathological of the spleen tissue, characterization with numerous abnormal enlargement cells [arrow indicate];
C) By immunohistochemistry, numerous infected hypertrophic cells were brown stained [arrow indicate].

Mandarin *S. chuatsi* has been evidenced a very high susceptible fish species for both freshwater ISKNV and marine water RSIV [Dong et al., 2010; Dong et al., 2008]. Spotted mandarin *S. scherzeri* is a distinct fish species in *Siniperca*. Until this report, natural outbreak of ISKNV/RSIV-like viral disease has been never documented, although an artificial infection from South Korea showed that spotted mandarin is susceptible to ISKNV from freshwater ornamental fish [Shin et al., 2014]. In China, in contrast to the large scale of artificial culture of mandarin *S. chuatsi* nationwide, much smaller scale of artificial culture spotted mandarin *S. scherzeri* was distributed mainly in Northeast China along Yalu River to Yellow Sea. As a result, outbreak of megalocytivirus-like disease in spotted mandarin might be ignored for a long time due to the small culture scale and relatively low influence. In this study, we confirmed the causative agent as an RSIV-type megalocytivirus in natural outbreak of spotted mandarin, which was strongly confirmed by PCR-based molecular identification, spleen tissue-based histopathology and IHC confirmation, cell culture-based virus isolation and IFA identification, and TEM observation. To the best of our knowledge, this is the first report of RSIV in spotted mandarin in China.

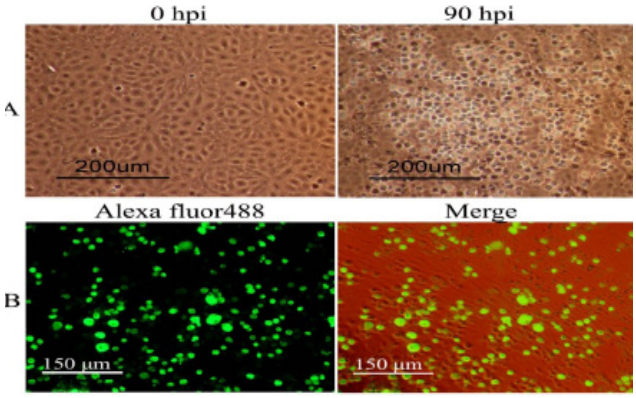

Figure 2: **Isolation and confirmation of KD1201 in MFF-1 cells.**

A) Typical CPEs, characterization with increasing rounding cells, were observed at 90 hours post infection.
B) IFA showed that numerous rounding cells were stained with strong green fluorescent under fluorescence microscope.

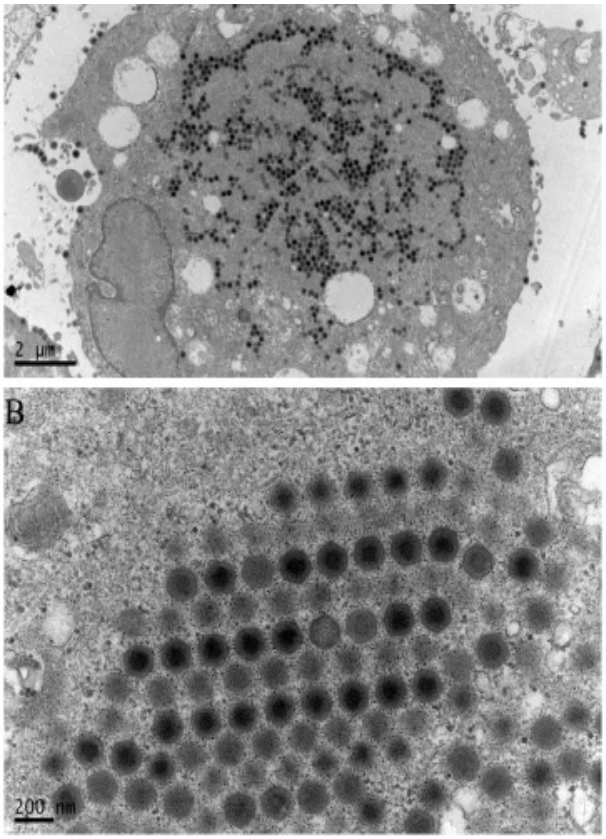

Figure 3: **Transmission electron micrographs of Megalocyti-KD1201 infected MFF-1 cells**

A) , numerous spherical viral particles distributing in the cytoplasm of infected MFF-1 cell, bar = 2 μm
B) Paracrystalline array of viral particles under a large magnification, bar = 200 nm

Megalocyti-KD1201 is an unusual RSIV isolate in freshwater fish

Sequence determination showed that the complete MCP of Megalocyti-KD1201 consists of 1362 nucleotides and encoding a 453 putative viral protein, which is consistent with those of other megalocytiviral isolates [Dong et al., 2010; Shinmoto et al., 2009]. Based on complete MCP nucleotide sequence, phylogenetic analysis showed that Megalocyti-KD1201 was classified into a distinct subgroup in RSIV clade, in which subgroup, an early RSIV isolate Ehime-1 from Japan was also included [Fig. 2]. Compared with the MCP from RSIV-Ehime-1, alignment showed that there are two nucleotides but one amino acid substitutions in that of Megalocyti-KD1201 [data not shown]. Previous studies classified wild megalocytiviral isolates into three clades, namely ISKNV, RSIV and TRBIV [Dong et al., 2010; Imajoh et al., 2007; Nakajima and Kunita, 2005; Shinmoto et al., 2009]. RSIV had the largest isolation numbers and consisted of two subtypes, namely RSIV-Ehime-1-like isolates and others [Dong et al., 2010; Imajoh et al., 2007]. RSIV-Ehime-1 was isolated from an early outbreak of RSIVD in Japan and used as the prepared viral strain for commercial RSIV vaccine [Mahardika et al., 2008; Nakajima et al., 1999; Nakajima et al., 1997]. According to literatures, RSIV-Ehime-1-like isolates had a small partial number in RSIV clade and was recorded rarely in other Asian country or region outside of Japan [Huang et al., 2011]. In this study, we identified Megalocyti-KD1201 as an Ehime-1-close RSIV, which indicates that this emerging RSIV in spotted mandarin in Northeast China was not spread from South China, the epicenter of ISKNV disease in cultured mandarin [Dong et al., 2008]. We speculated that Megalocyti-KD1201 is likely transmitted independently from marine fish along Yalu River estuary from Yellow Sea.

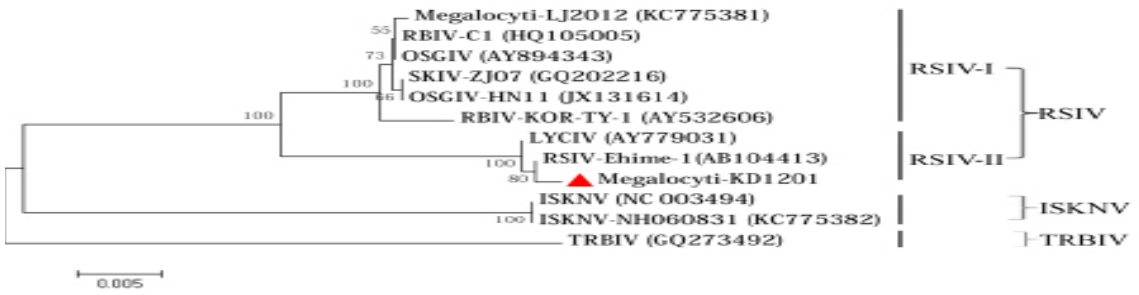

Figure 4: **Phylogenetic tree generated based on neighbor-joining analyses of the MCP gene sequences of various typical megalocytiviral isolates. Numbers at nodes indicate bootstrap confidence values [1000 replications]. Megalocyti-KD1201 is indicated in triangle.**

Inactivated vaccine is highly efficient to prevent RSIV diseases in cultured spotted mandarin

Based on Megalocyti-KD1201, formalin-killed whole cell vaccine was prepared and two field trial tests were conducted in a local cage-cultured spotted mandarin farm in Dandong since 2016-2018 [Table 1]. Before field test, the prepared vaccine were confirmed safe and effective in a mandarin model under laboratory condition as our previous report [Dong et al., 2013a]. In first field test, 186 of about 2,000 spotted mandarin juveniles [30-50 g/fish] were vaccinated and other 1,800 fish were used as non-vaccinated control. As was expected, outbreaks of RSIV diseases in non-vaccinated fish were observed from the second week of August in 2016. At that time, the cultured water temperature rose sharply to over 26-28 °C. During that outbreak, besides that ten fish were sacrificed for RSIV detection, the rest immunized 176 fish survived to commercial sizes after another 12 months breeding. By contrast, only less than 300 of 1800 non-immunized fish survived during RSIV outbreak. Similar high protection in immunized fish was also obtained from the second field test. Greater than 90% and less than 5% survivors were obtained in 4,000 vaccinated fish and 2,000 non-vaccinated control fish, respectively [Table 1]. All survived fish were cultured to market size and sold in 2018.

Table 1: Field trial tests of RSIV vaccine in cage-cultured spotted mandarin since 2016

Year	V or non-V	Fish numbers	Survival numbers within 18 months post vaccination	Survival (%) within 18 months post vaccination
2016	V	186	176[a]	>95%
	Non-V	1,800	≈300[b]	≈15%
2017	V	4,000	≈3,800	≈95%
	Non-V	2,000	< 100[b]	< 5%

V, Vaccination group;
Non-V, Non-vaccination group;

[a]Ten of 186 fish were sampled for RSIV detection at one month post vaccination at that time outbreak of RSIV occurred in non-immunization spotted mandarin. Other 176 fish were cultured to market size until 2015.

[b]both RSIVs were confirmed from moribund spotted mandarin by PCR-based molecular detection during outbreaks of RSIV diseases from August to September in 2016 and 2017.

From these two field tests, both results indicated that the inactivated RSIV vaccine in this study conferred effective protection against natural outbreak of RSIV diseases in cage cultured spotted mandarin and these results were also highly consistent with our

previous assessment of a field vaccination test in cultured mandarin in South China [Dong et al., 2013b]. It was reported that inactivated RSIV vaccine [Ehime-1 strain] in Japan showed significant efficacy in protecting various marine fish including yellow tail *Seriola quinqueradiata,* amberjack *S. dumerili,* kelp grouper *Epinephlelus moara*, striped jack *Pseudocaranx dentex* and spotted parrot fish *Oplegnathus punctatus* against challenge with RSIV [Nakajima et al., 2002]. According to a vaccine instruction, the licensed [Ehime-1 strain] RSIV vaccine is applied to three marine fish species including red seabream *Pagrus major,* Japanese amberjack *Seriola sp.* and striped jack *Pseudocaranx dentex* and freshwater fish is not included, although ISKNV-like virus was also recently confirmed from moribund mandarin in Japan [Tanaka et al., 2014]. In this study, Ehime-1-like RSIV was confirmed as the causative agent in natural mass mortality of cage-cultured spotted mandarin. Vaccinations from two field tests showed that the inactivated RSIV vaccine [Megalocyti-KD1201 strain] in this study conferred high efficacy to prevent natural outbreak of RSIV disease in cultured spotted mandarin. Collectively, this is the first confirmation of RSIV infection in cultured spotted mandarin, and vaccination of inactivated RSIV vaccine showed a promising treatment to prevent this RSIV disease. The findings will contribute a valuable application in spotted mandarin industry.

Acknowledgements

The research was supported by China Agriculture Research System (CARS-46-4B), Guangdong D&R plan project under No.2016B020202001 and Guangdong Provincial Special Fund for Modern Agriculture Industry Technology Innovation Teams (2019KJ141).

References

1. Dong C, Weng S, Luo Y, Huang M, Ai H, Yin Z, He J, 2010. A new marine megalocytivirus from spotted knifejaw, *Oplegnathus punctatus*, and its pathogenicity to freshwater mandarin fish, *Siniperca chuatsi.* Virus Res 147: 98-106.
2. Dong C, Weng S, Shi X, Xu X, Shi N, He J, 2008. Development of a mandarin fish *Siniperca chuatsi* fry cell line suitable for the study of infectious spleen and kidney necrosis virus [ISKNV]. Virus Res 135: 273-281.
3. Dong C, Xiong X, Luo Y, Weng S, Wang Q, He J, 2013a. Efficacy of a formalin-killed cell vaccine against infectious spleen and kidney necrosis virus [ISKNV] and immunoproteomic analysis of its major immunogenic proteins. Vet Microbiol 162: 419-428.
4. Dong Y, Weng S, He J, Dong C, 2013b. Field trial tests of FKC vaccines against RSIV genotype *Megalocytivirus* in cage-cultured mandarin fish [*Siniperca chuatsi*] in an inland reservoir. Fish Shellfish Immunol 35: 1598-1603.
5. Eaton HE, Metcalf J, Penny E, Tcherepanov V, Upton C, Brunetti CR, 2007. Comparative genomic analysis of the family Iridoviridae: re-annotating and defining the core set of iridovirus genes. Virol J 4, 11.
6. Go J, Lancaster M, Deece K, Dhungyel O, Whittington R, 2006. The molecular epidemiology of iridovirus in Murray cod [*Maccullochella peelii peelii*] and dwarf gourami [*Colisa lalia*] from distant biogeographical regions suggests a link between trade in ornamental fish and emerging iridoviral diseases. Mol Cell Probes 20: 212-222.
7. Huang SM, Tu C, Tseng CH, Huang CC, Chou CC, Kuo, H.C., Chang, S.K., 2011. Genetic analysis of fish iridoviruses isolated in Taiwan during 2001-2009. Arch Virol 156: 1505-1515.
8. Imajoh M, Ikawa T, Oshima S, 2007. Characterization of a new fibroblast cell line from a tail fin of red sea bream, *Pagrus major*, and phylogenetic relationships of a recent RSIV isolate in Japan. Virus Res 126: 45-52.
9. Kurita J, Nakajima K, 2012. Review: Megalocytiviruses. Viruses 4: 521-538.
10. Mahardika K, Haryanti Muzaki A, Miyazaki T, 2008. Histopathological and ultrastructural features of enlarged cells of humpback grouper *Cromileptes altivelis* challenged with Megalocytivirus [family Iridoviridae] after vaccination. Dis Aquat Organ 79: 163-168.
11. Nakajima K, Ito T, Kurita J, Kawakami H, Itano T, Fukuda Y, Aoi Y, Tooriyama T, Manabe S, 2002. Effectiveness of a Vaccine against Red Sea Bream Iridoviral Disease in Various Cultured Marine Fish under Laboratory Conditions. Fish Pathol 37: 90-91.
12. Nakajima K, Kunita J, 2005. [Red sea bream iridoviral disease]. Uirusu 55: 115-125.
13. Nakajima K, Maeno Y, Honda A, Yokoyama K, Tooriyama T, Manabe S, 1999. Effectiveness of a vaccine against red sea bream iridoviral disease in a field trial test. Dis Aquat Organ 36: 73-75.
14. Nakajima K, Maeno Y, Kurita J, Inui Y, 1997. Vaccination against Red Sea Bream Iridoviral Disease in Red Sea Bream Fish Pathol 32: 205-209.
15. Reed LJ, Muench H, 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27: 493-497.
16. Shin GW, White SL, Dahms HU, Jeong HD, Kim JH, 2014. Disease resistance and immune-relevant gene expression in golden mandarin fish, *Siniperca scherzeri* Steindachner, infected with infectious spleen and kidney necrosis virus-like agent. J Fish Dis 37: 1041-1054.
17. Shinmoto H, Taniguchi K, Ikawa T, Kawai K, Oshima S, 2009. Phenotypic diversity of infectious red sea bream iridovirus isolates from cultured fish in Japan. Appl Environ Microbiol 75: 3535-3541.
18. Shuang F, Luo Y, Xiong X, Weng S, Li Y, He J, Dong C, 2013. Virions proteins of an RSIV-type megalocytivirus from spotted knifejaw *Oplegnathus punctatus* [SKIV-ZJ07], Virology 437: 27-37.
19. Subramaniam K, Shariff M, Omar AR, Hair-Bejo, M, 2012. Megalocytivirus infection in fish. Reviews in Aquaculture, 221-233.
20. Sudthongkong C, Miyata M, Miyazaki T, 2002. Viral DNA sequences of genes encoding the ATPase and the major capsid protein of tropical iridovirus isolates which are pathogenic to

fishes in Japan, South China Sea and Southeast Asian countries. Arch Virol 147: 2089-2109.

21. Tamura K, Dudley J, Nei M, Kumar S, 2007. MEGA4: Molecular Evolutionary Genetics Analysis [MEGA] Software Version 4.0. Mol Biol Evol 24: 1596-1599.

22. Tanaka N, Izawa T, Kuwamura M, Higashiguchi N, Kezuka C, Kurata O, Wada S, Yamate J, 2014. The first case of infectious spleen and kidney necrosis virus [ISKNV] infection in aquarium-maintained mandarin fish, *Siniperca chuatsi* [Basilewsky], in Japan. J Fish Dis 37: 401-405.